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METHOD OF DIAGNOSING BREAST CANCER

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Respectfully submitted,

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DESCRIPTION

METHOD OF DIAGNOSING BREAST CANCER

TECHNICAL FIELD

The invention relates to methods of diagnosing breast cancer.

BACKGROUND OF THE INVENTION

Breast cancer is a complex disease in which numerous genetic changes. Little is known these abnormalities cause of breast tumorigenesis, although it has been reported that it occurred by a multistep process which can be broadly equated to transformation of normal cells via the steps of atypical ductal hyperplasia, ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC). There is evidence that only a proportion of premalignant lesions are committed to progression to invasive cancer while other lesions undergo spontaneous regression. The explanation of molecular participation, which leads to development of primary breast cancer, its progression, and its formation of metastasis, is the main focus for new strategies targeted at prevention and treatment.

Gene-expression profiles generated by cDNA microarray analysis can provide considerably more detail about the nature of individual cancers than traditional histopathological methods are able to supply. The promise of such information lies in its potential for improving clinical strategies for treating neoplastic diseases and developing novel drugs (Petricoin, E. F., 3rd, Hackett, J. L., Lesko, L. J., Puri, R. K., Gutman, S. I., Chumakov, K., Woodcock, J., Feigal, D. W., Jr., Zoon, K. C., and Sistare, F. D. Medical applications of microarray technologies: a regulatory science perspective. Nat Genet, *32 Suppl:* 474-479, 2002.). To aim this goal, we have been analyzing the expression profiles of tumor or tumors from various tissues by cDNA microarrays (Okabe, H.et al., Genome-wide analysis of gene expression in human hepatocellular carcinomas using cDNA microarray: identification of genes involved in viral carcinogenesis and tumor progression. Cancer Res, *61:* 2129-2137, 2001.; Hasegawa, S.et al.,Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23,040 genes. Cancer Res, *62:* 7012-7017, 2002.; Kaneta, Y.et al., and Ohno, R. Prediction of Sensitivity to STI571 among Chronic Myeloid Leukemia Patients by Genome-wide cDNA

Microarray Analysis. Jpn J Cancer Res, *93:* 849-856, 2002.; Kaneta, Y.et al., Genome-wide analysis of gene-expression profiles in chronic myeloid leukemia cells using a cDNA microarray. Int J Oncol, *23:* 681-691, 2003.; Kitahara, O.et al., Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia. Cancer Res, *61:* 3544-3549, 2001.; Lin, Y. et al. Molecular diagnosis of colorectal tumors by expression profiles of 50 genes expressed differentially in adenomas and carcinomas. Oncogene, *21:* 4120-4128, 2002.; Nagayama, S. et al., Genome-wide analysis of gene expression in synovial sarcomas using a cDNA microarray. Cancer Res, *62:* 5859-5866, 2002.; Okutsu, J. et al., Prediction of chemosensitivity for patients with acute myeloid leukemia, according to expression levels of 28 genes selected by genome-wide complementary DNA microarray analysis. Mol Cancer Ther, *1:* 1035-1042, 2002.; Kikuchi, T. et al., Expression profiles of non-small cell lung cancers on cDNA microarrays: identification of genes for prediction of lymph-node metastasis and sensitivity to anti-cancer drugs. Oncogene, *22:* 2192-2205, 2003.).

Recent analysis of the levels of expression of thousands of genes with the use of cDNA microarrays has been reported distinct patterns in different types of breast cancer (Sgroi, D. C.et al., In vivo gene expression profile analysis of human breast cancer progression. Cancer Res, *59:* 5656-5661, 1999.; Sorlie, T.et al., Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A, *98:* 10869-10874, 2001.; Kauraniemi, P.et al., New amplified and highly expressed genes discovered in the ERBB2 amplicon in breast cancer by cDNA microarrays. Cancer Res, *61:* 8235-8240, 2001.; Gruvberger, S.et al., S. Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. Cancer Res, *61:* 5979-5984, 2001.; Dressman, M. et al., Gene expression profiling detects gene amplification and differentiates tumor types in breast cancer. Cancer Res, *63:* 2194-2199, 2003.).

Some studies of gene-expression profiles of breast cancers have discovered genes that might be candidates as diagnostic markers or prognosis profiles. However, these data derived from tumor masses cannot adequately reflect expressional changes during breast carcinogenesis, because breast cancer cells exist as a solid mass with a highly inflammatory reaction and containing various cellular components. Therefore, previous published microarray data are likely to reflect heterogenous profiles.

Studies designed to reveal mechanisms of carcinogenesis have already facilitated

identification of molecular targets for anti-tumor agents. For example, inhibitors of farnexyltransferase (FTIs) which were originally developed to inhibit the growth-signaling pathway related to Ras, whose activation depends on posttranslational farnesylation, has been effective in treating Ras-dependent tumors in animal models (He et al., Cell 99:335-45 (1999)). Clinical trials on human using a combination or anti-cancer drugs and anti-HER2 monoclonal antibody, trastuzumab, have been conducted to antagonize the proto-oncogene receptor HER2/neu; and have been achieving improved clinical response and overall survival of breast-cancer patients (Lin et al., Cancer Res 61:6345-9 (2001)). A tyrosine kinase inhibitor, STI-571, which selectively inactivates bcr-abl fusion proteins, has been developed to treat chronic myelogenous leukemias wherein constitutive activation of bcr-abl tyrosine kinase plays a crucial role in the transformation of leukocytes. Agents of these kinds are designed to suppress oncogenic activity of specific gene products (Fujita et al., Cancer Res 61:7722-6 (2001)). Therefore, gene products commonly up-regulated in cancerous cells may serve as potential targets for developing novel anti-cancer agents.

It has been demonstrated that CD8+ cytotoxic T lymphocytes (CTLs) recognize epitope peptides derived from tumor-associated antigens (TAAs) presented on MHC Class I molecule, and lyse tumor cells. Since the discovery of MAGE family as the first example of TAAs, many other TAAs have been discovered using immunological approaches (Boon, Int J Cancer 54: 177-80 (1993); Boon and van der Bruggen, J Exp Med 183: 725-9 (1996); van der Bruggen et al., Science 254: 1643-7 (1991); Brichard et al., J Exp Med 178: 489-95 (1993); Kawakami et al., J Exp Med 180: 347-52 (1994)). Some of the discovered TAAs are now in the stage of clinical development as targets of immunotherapy. TAAs discovered so far include MAGE (van der Bruggen et al., Science 254: 1643-7 (1991)), gp100 (Kawakami et al., J Exp Med 180: 347-52 (1994)), SART (Shichijo et al., J Exp Med 187: 277-88 (1998)), and NY-ESO-1 (Chen et al., Proc Natl Acad Sci USA 94: 1914-8 (1997)). On the other hand, gene products which had been demonstrated to be specifically overexpressed in tumor cells, have been shown to be recognized as targets inducing cellular immune responses. Such gene products include p53 (Umano et al., Brit J Cancer 84: 1052-7 (2001)), HER2/neu (Tanaka et al., Brit J Cancer 84: 94-9 (2001)), CEA (Nukaya et al., Int J Cancer 80: 92-7 (1999)), and so on.

In spite of significant progress in basic and clinical research concerning TAAs (Rosenbeg et al., Nature Med 4: 321-7 (1998); Mukherji et al., Proc Natl Acad Sci USA 92:

8078-82 (1995); Hu et al., Cancer Res 56: 2479-83 (1996)), only limited number of candidate TAAs for the treatment of adenocarcinomas, including colorectal cancer, are available. TAAs abundantly expressed in cancer cells, and at the same time which expression is restricted to cancer cells would be promising candidates as immunotherapeutic targets. Further, identification of new TAAs inducing potent and specific antitumor immune responses is expected to encourage clinical use of peptide vaccination strategy in various types of cancer (Boon and can der Bruggen, J Exp Med 183: 725-9 (1996); van der Bruggen et al., Science 254: 1643-7 (1991); Brichard et al., J Exp Med 178: 489-95 (1993); Kawakami et al.,J Exp Med 180: 347-52 (1994); Shichijo et al., J Exp Med 187: 277-88 (1998); Chen et al., Proc Natl Acad Sci USA 94: 1914-8 (1997); Harris, J Natl Cancer Inst 88: 1442-5 (1996); Butterfield et al., Cancer Res 59: 3134-42 (1999); Vissers et al., Cancer Res 59: 5554-9 (1999); van der Burg et al., J Immunol 156: 3308-14 (1996); Tanaka et al., Cancer Res 57: 4465-8 (1997); Fujie et al., Int J Cancer 80: 169-72 (1999); Kikuchi et al., Int J Cancer 81: 459-66 (1999); Oiso et al., Int J Cancer 81: 387-94 (1999)).

It has been repeatedly reported that peptide-stimulated peripheral blood mononuclear cells (PBMCs) from certain healthy donors produce significant levels of IFN-γ in response to the peptide, but rarely exert cytotoxicity against tumor cells in an HLA-A24 or –A0201 restricted manner in ^{51}Cr-release assays (Kawano et al., Cance Res 60: 3550-8 (2000); Nishizaka et al., Cancer Res 60: 4830-7 (2000); Tamura et al., Jpn J Cancer Res 92: 762-7 (2001)). However, both of HLA-A24 and HLA-A0201 are one of the popular HLA alleles in Japanese, as well as Caucasian (Date et al., Tissue Antigens 47: 93-101 (1996); Kondo et al., J Immunol 155: 4307-12 (1995); Kubo et al., J Immunol 152: 3913-24 (1994); Imanishi et al., Proceeding of the eleventh International Hictocompatibility Workshop and Conference Oxford University Press, Oxford, 1065 (1992); Williams et al., Tissue Antigen 49: 129 (1997)). Thus, antigenic peptides of carcinomas presented by these HLAs may be especially useful for the treatment of carcinomas among Japanese and Caucasian. Further, it is known that the induction of low-affinity CTL in vitro usually results from the use of peptide at a high concentration, generating a high level of specific peptide/MHC complexes on antigen presenting cells (APCs), which will effectively activate these CTL (Alexander-Miller et al., Proc Natl Acad Sci USA 93: 4102-7 (1996)).

SUMMARY OF THE INVENTION

The invention is based on the discovery of a pattern of gene expression correlated with breast cancer (BRC). The genes that are differentially expressed in breast cancer are collectively referred to herein as "BRC nucleic acids" or "BRC polynucleotides" and the corresponding encoded polypeptides are referred to as "BRC polypeptides" or "BRC proteins."

Accordingly, the invention features a method of diagnosing or determining a predisposition to breast cancer in a subject by determining an expression level of a BRC-associated gene in a patient derived biological sample, such as tissue sample. By BRC-associated gene is meant a gene that is characterized by an expression level which differs in a cell obtained from a BRC cell compared to a normal cell. A normal cell is one obtained from breast tissue. A BRC-associated gene is one or more gene listed in tables 3-8. An alteration, *e.g.*, increase or decrease of the level of expression of the gene compared to a normal control level of the gene indicates that the subject suffers from or is at risk of developing BRC.

By normal control level is meant a level of gene expression detected in a normal, healthy individual or in a population of individuals known not to be suffering from breast cancer. A control level is a single expression pattern derived from a single reference population or from a plurality of expression patterns. For example, the control level can be a database of expression patterns from previously tested cells. A normal individual is one with no clinical symptoms of breast cancer.

An increase in the level of BRC-associated genes listed in tables 3, 5, and 7 detected in a test sample compared to a normal control level indicates the subject (from which the sample was obtained) suffers from or is at risk of developing BRC. In contrast, a decrease in the level of BRC-associated genes listed in tables 4, 6, and 8 detected in a test sample compared to a normal control level indicates said subject suffers from or is at risk of developing BRC.

Alternatively, expression of a panel of BRC-associated genes in the sample is compared to a BRC control level of the same panel of genes. By BRC control level is meant the expression profile of the BRC-associated genes found in a population suffering from BRC.

Gene expression is increased or decreased 10%, 25%, 50% compared to the control

level. Alternately, gene expression is increased or decreased 1, 2, 5 or more fold compared to the control level. Expression is determined by detecting hybridization, *e.g.*, on an array, of a BRC-associated gene probe to a gene transcript of the patient-derived tissue sample.

The patient derived tissue sample is any tissue from a test subject, *e.g.*, a patient known to or suspected of having BRC. For example, the tissue contains an epithelial cell. For example, the tissue is an epithelial cell from a breast ductal carcinoma.

The invention also provides a BRC reference expression profile of a gene expression level of two or more of BRC-associated genes listed in tables 3-8. Alternatively, the invention provides a BRC reference expression profile of the levels of expression two or more of BRC-associated genes listed in tables 3, 5, and 7 or BRC-associated genes listed in tables 4, 6, and 8.

The invention further provides methods of identifing an agent that inhibits or enhances the expression or activity of a BRC-associated gene, *e.g.* BRC-associated genes listed in tables 3, 5, and 7 by contacting a test cell expressing a BRC-associated gene with a test compound and determining the expression level of the BRC associated gene. The test cell is a epithelial cell such as an epithelial cell from a breast carcinoma. A decrease of the level compared to a normal control level of the gene indicates that the test agent is an inhibitor of the BRC-associated gene and reduces a symptom of BRC, *e.g.* BRC-associated genes listed in tables 3, 5, and 7. Alternatively, an increase of the level or activity compared to a normal control level or activity of the gene indicates that said test agent is an enhancer of expression or function of the BRC-associated gene and reduces a symptom of BRC, *e.g*, BRC-associated genes listed in tables 4, 6, and 8.

The invention also provides a kit with a detection reagent which binds to one or more BRC nucleic acids or which binds to a gene product encoded by the nucleic acid sequences. Also provided is an array of nucleic acids that binds to one or more BRC nucleic acids.

Therapeutic methods include a method of treating or preventing BRC in a subject by administering to the subject an antisense composition. The antisense composition reduces the expression of a specific target gene, *e.g.*, the antisense composition contains a nucleotide, which is complementary to a sequence selected from the group consisting of BRC-associated genes listed in tables 3, 5, and 7. Another method includes the steps of administering to a subject an short interfering RNA (siRNA) composition. The siRNA composition reduces the expression of a nucleic acid selected from the group consisting of BRC-associated genes

listed in tables 3, 5, and 7. In yet another method, treatment or prevention of BRC in a subject is carried out by administering to a subject a ribozyme composition. The nucleic acid-specific ribozyme composition reduces the expression of a nucleic acid selected from the group consisting of BRC-associated genes listed in tables 3, 5, and 7. Other therapeutic methods include those in which a subject is administered a compound that increases the expression of BRC-associated genes listed in tables 4, 6, and 8 or activity of a polypeptide encoded by BRC-associated genes listed in tables 4, 6, and 8.

The invention also includes vaccines and vaccination methods. For example, a method of treating or preventing BRC in a subject is carried out by administering to the subject a vaccine containing a polypeptide encoded by a nucleic acid selected from the group consisting of BRC-associated genes listed in tables 3, 5, and 7 or an immunologically active fragment such a polypeptide. An immunologically active fragment is a polypeptide that is shorter in length than the full-length naturally-occurring protein and which induces an immune response. For example, an immunologically active fragment at least 8 residues in length and stimulates an immune cell such as a T cell or a B cell. Immune cell stimulation is measured by detecting cell proliferation, elaboration of cytokines (*e.g.*, IL-2), or production of an antibody.

Alternatively, the present invention provides target molecules for treating or preventing metastasis of breast cancer. According to the present invention, genes listed in table 11 were identified as genes that showed unique altered expression patterns in breast cancer cells with lymph-node metastasis. Thus, metastasis of breast cancer can be treated or prevented via the suppression of the expression or activity of up-regulated genes selected from the group consisting of VAMP3, MGC11257, GSPT1, DNM2, CFL1, CLNS1A, SENP2, NDUFS3, NOP5/NOP58, PSMD13, SUOX, HRB2, LOC154467, THTPA, ZRF1, LOC51255, DEAF1, NEU1, UGCGL1, BRAF, TUFM, FLJ10726, DNAJB1, AP4S1, and MRPL40. Moreover, metastasis of breast cancer can also be treated or prevented through enhancing the expression or activity of UBA52, GenBank Acc# AA634090, CEACAM3, C21orf97, KIAA1040, EEF1D, FUS, GenBank Acc# AW965200, and KIAA0475 in cancerous cells.

The present invention also provides methods for predicting metastasis of breast cancer. The method comprises the step of measuring the expression level of marker genes selected from the group consisting of genes listed in table 11. The marker genes were

identified as genes that show unique altered expression patterns in breast cancer cells of patients with lynphnode metastasis. Therefore, metastasis of the breast cancer in a subject can be predicted by determining whether the expression level detected in a sample derived from the subject is closer to the mean expression level of lynphnode metastasis positive cases or negative cases in reference samples.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

One advantage of the methods described herein is that the disease is identified prior to detection of overt clinical symptoms of breast cancer. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1

Microdissection of DCIS, IDC cells and normal breast ductal epithelial cells using Laser microbeam microdissection (LMM). DCIS cells (10326T case), and IDC cells (10502T), and normal breast ductal epithelial cell (10341N) from each specimen were microdissected from hematoxylin and eosin stained sections. Images of premicrodissected (lane A), postmicrodissected (lane B), and the microdissected cells (lane C) are shown.

Figure 2

Unsupervised two-dimensional hierarchical clustering analysis of 710 genes across 102 samples. (A) Each horizontal row represents a breast cancer patient, and each vertical column shows a single gene. The color of each well represents with red and green indicating transcript levels respectively above and below the median for that gene across all samples. An asterisk mark shows major historical type, and a sharp mark shows minor historical type in same case. Red square indicates one duplicated cases (10149a1 and

10149a1T). Black, unchanged expression. ER, ER status measured by EIA; LN, lymph-node metastasis status, ESR1, expression profiles of ESR1 in this microarray. (B) Two-dimensional hierarchical clustering analysis of 89 genes across 16 samples with 2 differentiated lesion microdissected from 8 breast cancer patients. (C) Clustering analysis using 25 genes that showed differentially expression between well- or poorly-differentiated invasive ductal cancer cells.

Figure 3

Supervised hierarchical clustering analysis of genes using 97 genes selected by a random-permutation test. In the horizontal row, 41 ER-positive samples and 28 negative samples (selected from premenopausal patients), In the vertical column, 97 genes were clustered in different branches according to similarity in relative expression ratios. Genes in the lower main branch were preferentially expressed similarly to the expression level of ESR1 as well as Figure 1A. Those in the upper branch were in inverse proportion of ESR1.

Figure 4

Genes with altered expression in DCIS relative to normal duct and in IDC relative to DCIS. (A) Cluster of 251genes which commonly up- or down-regulated in DCIS and IDC. (B) Cluster of 74 genes with elevated or decreased expression from DCIS to IDC. (C) Cluster of 65 genes which specifically up- or down-regulated in IDC.

Figure 5

Semi-quatitative RT-PCR validation of highly expressed genes, expression of 5 genes (AI261804, AA205444 and AA167194 in well-differentiated 12 cases, and AA676987 and H22566 in poorly -differentiated 12 cases) and GAPDH (internal control) was examined by semi-quantitative RT-PCR. Signals of the microarray corresponded to the results of semi-quantitative RT-PCR experiments. Normal breast duct cells was prepared from normal ductal epithelial cells in premenopausal 15 patients used in this microarray. MG, whole human mammary gland.

Figure 6

Supervised hierarchical clustering analysis of genes using 206 genes selected by a random-permutation test, in the horizontal row, 69 samples (selected from IDC patients). In the vertical column, 97 genes were clustered in different branches according to

similarity in relative expression ratios. Genes in the blue and red-bar branch were preferentially expressed similarly to the expression level of poorly-differentiated type and well-differentiated type.

Figure 7

(A) Result of a two-dimensional hierarchical clustering analysis using 34 genes selected by evaluation of classification and leave-one-out test after a random-permutation test for establishing a predictive scoring system. Genes in the upper main branch were preferentially expressed in cases involving lymph node metastasis; those in the lowerr branch were more highly expressed in lymph node-negative cases.

(B) Strength of genes appearing in (A) for separating non-metastatic (lymph node-negative) tumors from metastatic (lymph node-positive) tumors. Blue square represent node-positive cases; red denotes negative cases. The 20 sky-blue square is a lymph node-positive test case and the 17 yellow triangle represents lymph node-negative test cases that had not been used for establishing prediction scores.

(C) Correlation between the prediction score for metastasis and clinical information after operation.

DETAILED DESCRIPTION

Generally breast cancer cells exist as a solid mass with a highly inflammatory reaction and containing various cellular components. Therefore, previous published microarray data are likely to reflect heterogenous profiles.

With these issues in view, the present inventers prepared purified populations of breast cancer cells and normal breast epithelial duct cells by a method of laser-microbeam microdissection (LMM), and analyzed genome-wide gene-expression profiles of 81 BRCs including 12 ductal carcinomas in situ (DCIS) and 69 invasive ductal carcinomas (IDC) using a cDNA microarray representing 23,040 genes. These data should provide not only important information about breast carcinogenesis, but should identify candidate genes whose products might serve as diagnostic markers and/or as molecular targets for treatment of many patients with breast cancer and provides clinically relevant information.

The present invention is based in part on the discovery of changes in expression patterns of multiple nucleic acids in epithelial cells from carcinomas of patients with BRC.

The differences in gene expression were identified by using a comprehensive cDNA microarray system.

The gene-expression profiles of cancer cells from 81 BRCs including 12 DCISs and 69 IDCs were analyzed using cDNA microarray representing 23,040 genes couples with laser microdissection. By comparing expression patterns between cancer cells from diagnostic BRC patients and normal ductal epithelial cells purely selected with Laser Microdisection, 102 genes (shown in tables 3, 5 and 7) were identified as commonly up-regulated in BRC cells, and 288 genes (shown in tables 4, 6 and 8) were identified as being commonly down-regulated in BRC cells. In addition, selection was made of candidate molecular markers with the potential of detecting cancer-related proteins in serum or sputum of patients, and discovered some potential targets for development of signal-suppressing strategies in human BRC. Among them, genes listed in table 3 and table 4 show list of genes with altered expression between BRC including DCIS and IDC, and nomal tissue. Genes commonly up- or down- regulated in DCIS and IDC are shown in table 3 and table 4 respectively. Genes with elevated or decreasd expression from DCIS to IDC are also listed in table 5 and table 6 respectively. Furthermore, genes commonly up- or down- regulated in IDC comparing with nomal tissue are listed in table 7 and table 8 respectively.

The differentially expressed genes identified herein are used for diagnostic purposes as markers of BRC and as gene targets, the expression of which is altered to treat or alleviate a symptom of BRC. Alternatively, the differentially expressed genes within DCIS and IDC identified herein are used for diagnostic purposes as markers to distinguish IDC from DCIS and as gene targets, the expression of which is altered to treat or alleviate a symptom of IDC.

The genes whose expression levels are modulated (*i.e.*, increased or decreased) in BRC patients are summarized in Tables 3-8 and are collectively referred to herein as " BRC-associated genes " , "BRC nucleic acids" or "BRC polynucleotides" and the corresponding encoded polypeptides are referred to as "BRC polypeptides" or "BRC proteins." Unless indicated otherwise, "BRC" is meant to refer to any of the sequences disclosed herein. (*e.g.*, BRC-associated genes listed in tables 3-8). The genes have been previously described and are presented along with a database accession number.

By measuring expression of the various genes in a sample of cells, BRC is diagnosed. Similarly, measuring the expression of these genes in response to various agents can identify agents for treating BRC.

The invention involves determining (*e.g.*, measuring) the expression of at least one, and up to all the BRC-associated genes listed in Tables 3-8. Using sequence information provided by the GenBank™ database entries for the known sequences the BRC-associated genes are detected and measured using techniques well known to one of ordinary skill in the art. For example, sequences within the sequence database entries corresponding to BRC-associated genes, are used to construct probes for detecting BRC RNA sequences in, *e.g.*, northern blot hybridization analysis. Probes include at least 10, 20, 50, 100, 200 nucleotides of a reference sequence. As another example, the sequences can be used to construct primers for specifically amplifying the BRC nucleic acid in, *e.g*, amplification-based detection methods such as reverse-transcription based polymerase chain reaction.

Expression level of one or more of the BRC-associated genes in the test cell population, *e.g.*, a patient derived tissues sample, is then compared to expression levels of the some genes in a reference population. The reference cell population includes one or more cells for which the compared parameter is known, *i.e.*, breast ductal carcinoma cells or normal breast ductal epithelial cells.

Whether or not a pattern of gene expression levels in the test cell population compared to the reference cell population indicates BRC or predisposition thereto depends upon the composition of the reference cell population. For example, if the reference cell population is composed of non-BRC cells, a similar gene expression pattern in the test cell population and reference cell population indicates the test cell population is non-BRC. Conversely, if the reference cell population is made up of BRC cells, a similar gene expression profile between the test cell population and the reference cell population indicates that the test cell population includes BRC cells.

A level of expression of a BRC marker gene in a test cell population is considered altered in levels of expression if its expression level varies from the reference cell population by more than 1.0, 1.5, 2.0, 5.0, 10.0 or more fold from the expression level of the corresponding BRC marker gene in the reference cell population.

Differential gene expression between a test cell population and a reference cell population is normalized to a control nucleic acid, *e.g.* a housekeeping gene. For example, a control nucleic acid is one which is known not to differ depending on the cancerous or non-cancerous state of the cell. Expression levels of the control nucleic acid in the test and reference nucleic acid can be used to normalize signal levels in the compared populations.

Control genes include, *e.g.*. β-actin, glyceraldehyde 3- phosphate dehydrogenase or ribosomal protein P1.

The test cell population is compared to multiple reference cell populations. Each of the multiple reference populations may differ in the known parameter. Thus, a test cell population may be compared to a second reference cell population known to contain, *e.g.*, BRC cells, as well as a second reference population known to contain, *e.g.*, non-BRC cells (normal cells). The test cell is included in a tissue type or cell sample from a subject known to contain, or to be suspected of containing, BRC cells.

The test cell is obtained from a bodily tissue or a bodily fluid, *e.g.*, biological fluid (such as blood or sputum). For example, the test cell is purified from breast tissue. Preferably, the test cell population comprises an epithelial cell. The epithelial cell is from tissue known to be or suspected to be a breast ductal carcinoma.

Cells in the reference cell population are derived from a tissue type as similar to test cell. Optionally, the reference cell population is a cell line, *e.g.* a BRC cell line (positive control) or a normal non-BRC cell line (negative control). Alternatively, the control cell population is derived from a database of molecular information derived from cells for which the assayed parameter or condition is known.

The subject is preferably a mammal. The mammal can be, *e.g.*, a human, non-human primate, mouse, rat, dog, cat, horse, or cow.

Expression of the genes disclosed herein is determined at the protein or nucleic acid level using methods known in the art. For example, Northern hybridization analysis using probes which specifically recognize one or more of these nucleic acid sequences can be used to determine gene expression. Alternatively, expression is measured using reverse-transcription-based PCR assays, *e.g.*, using primers specific for the differentially expressed gene sequences. Expression is also determined at the protein level, *i.e.*, by measuring the levels of polypeptides encoded by the gene products described herein, or biological activity thereof. Such methods are well known in the art and include, e.g., immunoassays based on antibodies to proteins encoded by the genes. The biological activity of the proteins encoded by the genes are also well known.

Diagnosing breast cancer

BRC is diagnosed by measuring the level of expression of one or more BRC nucleic acids from a test population of cells, (*i.e.*, a patient derived biological sample). Preferably, the test cell population contains an epithelial cell, *e.g.*, a cell obtained from breast tissue. Gene expression is also measured from blood or other bodily fluids such as urine. Other biological samples can be used for measuring the protein level. For example, the protein level in the blood, serum derived from subject to be diagnosed can be measured by immunoassay or biological assay.

Expression of one or more BRC-associated genes, *e.g.*, genes listed in tables 3-8 is determined in the test cell or biological sample and compared to the expression of the normal control level. A normal control level is an expression profile of a BRC-associated gene typically found in a population known not to be suffering from BRC. An increase or a decrease of the level of expression in the patient derived tissue sample of the BRC-associated genes indicates that the subject is suffering from or is at risk of developing BRC. For example, an increase in expression of BRC-associated genes listed in tables 3, 5, and 7 in the test population compared to the normal control level indicates that the subject is suffering from or is at risk of developing BRC. Conversely, a decrease in expression of BRC-associated genes listed in tables 4, 6, and 8 in the test population compared to the normal control level indicates that the subject is suffering from or is at risk of developing BRC.

When one or more of the BRC-associated genes are altered in the test population compared to the normal control level indicates that the subject suffers from or is at risk of developing BRC. For example, at least 1%, 5%, 25%, 50%, 60%, 80%, 90% or more of the panel of BRC-associated genes (genes listed in tables 3-8) are altered.

Identifying histopathological differentiation of BRC

The present invention provides a method for identifying histopathological differentiation of BRC in a subject, the method comprising the steps of:

(a) detecting an expression level of one or more marker genes in a tissue sample collected from a subject to be predicted, wherein the one or more marker genes are selected from the group consisting of genes listed in tables 1 and 10; and

(b) comparing the expression level of the one or more marker genes to that of a well-differentiated and poorly-differentiated cases; and

(c) when the expression level of one or marker genes is close to that of the well-differentiated case, determining the tissue sample was well-differentiated and

when the expression level of one or marker genes is close to that of the poorly-differentiated case, determining the tissue sample was poorly-differentiated.

In the present invention, marker gene(s) for identifying histopathological differentiation of BRC may be at least one gene selected from the group consisting of 231 genes shown in Tables 1 and 10. The nucleotide sequences of the genes and amino acid sequences encoded thereby are known in the art. See Tables 1 and 10 for the Accession Numbers of the genes.

Identifying Agents that inhibit or enhance BRC-associated gene expression

An agent that inhibits the expression or activity of a BRC-associated gene is identified by contacting a test cell population expressing a BRC-associated up-regulated gene with a test agent and determining the expression level of the BRC-associated gene. A decrease in expression in the presence of the agent compared to the normal control level (or compared to the level in the absence of the test agent) indicates the agent is an inhibitor of a BRC-associated up-regulated gene and useful to inhibit BRC.

Alternatively, an agent that enhances the expression or activity of a BRC-associated down-regulated gene is identified by contacting a test cell population expressing a BRC-associated gene with a test agent and determining the expression level or activity of the BRC-associated down-regulated gene. An increase of expression or activity compared to a normal control expression level or activity of the BRC-associated gene indicates that the test agent augments expression or activity of the BRC-associated down-regulated gene.

The test cell population is any cell expressing the BRC-associated genes. For example, the test cell population contains an epithelial cell, such as a cell is or derived from breast tissue. For example, the test cell is an immortalized cell line derived from an carcinoma cell. Alternatively, the test cell is a cell, which has been transfected with a BRC-associated gene or which has been transfected with a regulatory sequence (*e.g.* promoter sequence) from a BRC-associated gene operably linked to a reporter gene.

Assessing efficacy of treatment of BRC in a subject

The differentially expressed BRC-associated gene identified herein also allow for the course of treatment of BRC to be monitored. In this method, a test cell population is provided from a subject undergoing treatment for BRC. If desired, test cell populations are obtained from the subject at various time points before, during, or after treatment. Expression of one

or more of the BRC-associated gene, in the cell population is then determined and compared to a reference cell population which includes cells whose BRC state is known. The reference cells have not been exposed to the treatment.

If the reference cell population contains no BRC cells, a similarity in expression between BRC-associated gene in the test cell population and the reference cell population indicates that the treatment is efficacious. However, a difference in expression between BRC-associated gene in the test population and a normal control reference cell population indicates a less favorable clinical outcome or prognosis.

By "efficacious" is meant that the treatment leads to a reduction in expression of a pathologically up-regulated gene, increase in expression of a pathologically down-regulated gene or a decrease in size, prevalence, or metastatic potential of breast ductal carcinoma in a subject. When treatment is applied prophylactically, "efficacious" means that the treatment retards or prevents a breast tumor from forming or retards, prevents, or alleviates a symptom of clinical BRC. Assessment of breast tumors is made using standard clinical protocols.

Efficaciousness is determined in association with any known method for diagnosing or treating BRC. BRC is diagnosed for example, by identifying symptomatic anomalies, *e.g.*, weight loss, abdominal pain, back pain, anorexia, nausea, vomiting and generalized malaise, weakness, and jaundice.

Selecting a therapeutic agent for treating BRC that is appropriate for a particular individual

Differences in the genetic makeup of individuals can result in differences in their relative abilities to metabolize various drugs. An agent that is metabolized in a subject to act as an anti-BRC agent can manifest itself by inducing a change in gene expression pattern in the subject's cells from that characteristic of a cancerous state to a gene expression pattern characteristic of a non-cancerous state. Accordingly, the differentially expressed BRC-associated gene disclosed herein allow for a putative therapeutic or prophylactic inhibitor of BRC to be tested in a test cell population from a selected subject in order to determine if the agent is a suitable inhibitor of BRC in the subject.

To identify an inhibitor of BRC, that is appropriate for a specific subject, a test cell population from the subject is exposed to a therapeutic agent, and the expression of one or more of BRC-associated genes listed in table 3-8 is determined.

The test cell population contains a BRC cell expressing a BRC-associated gene. Preferably, the test cell is an epithelial cell. For example a test cell population is incubated in the presence of a candidate agent and the pattern of gene expression of the test sample is measured and compared to one or more reference profiles, *e.g.*, a BRC reference expression profile or a non-BRC reference expression profile.

A decrease in expression of one or more of BRC-associated genes listed in tables 3, 5, and 7 or an increase in expression of one or more of BRC-associated genes listed in tables 4, 6, and 8 in a test cell population relative to a reference cell population containing BRC is indicative that the agent is therapeutic.

The test agent can be any compound or composition. For example, the test agents are immunomodulatory agents.

Screening assays for identifying therapeutic agents

The differentially expressed genes disclosed herein can also be used to identify candidate therapeutic agents for treating BRC. The method is based on screening a candidate therapeutic agent to determine if it converts an expression profile of BRC-associated genes listed in tables 3-8 characteristic of a BRC state to a pattern indicative of a non-BRC state.

In the method, a cell is exposed to a test agent or a combination of test agents (sequentially or consequentially) and the expression of one or more BRC-associated genes listed in tables 3-8 in the cell is measured. The expression profile of the BRC-associated gene in the test population is compared to expression level of the BRC-associated gene in a reference cell population that is not exposed to the test agent.

An agent effective in stimulating expression of under-expressed genes, or in suppressing expression of over-expressed genes is deemed to lead to a clinical benefit such compounds are further tested for the ability to prevent breast ductal carcinomal growth in animals or test subjects.

In a further embodiment, the present invention provides methods for screening candidate agents which are potential targets in the treatment of BRC. As discussed in detail above, by controlling the expression levels or activities of marker genes, one can control the onset and progression of BRC. Thus, candidate agents, which are potential targets in the treatment of BRC, can be identified through screenings that use the expression levels and activities of marker genes as indices. In the context of the present invention, such screening

may comprise, for example, the following steps:

a) contacting a test compound with a polypeptide encoded by a polynucleotide selected from the group consisting of the genes listed in table 3, 4, 5, 6, 7 or 8;

b) detecting the binding activity between the polypeptide and the test compound; and

c) selecting a compound that binds to the polypeptide.

Alternatively, the screening method of the present invention may comprise the following steps:

a) contacting a candidate compound with a cell expressing one or more marker genes, wherein the one or more marker genes is selected from the group consisting of the genes listed in table 3, 4, 5, 6, 7 or 8; and

b) selecting a compound that reduces the expression level of one or more marker genes selected from the group consisting of the genes listed in table 3, 5, and 7, or elevates the expression level of one or more marker genes selected from the group consisting of the genes listed in table 4, 6 and 8.

Cells expressing a marker gene include, for example, cell lines established from BRC; such cells can be used for the above screening of the present invention.

Alternatively, the screening method of the present invention may comprise the following steps:

a) contacting a test compound with a polypeptide encoded by a polynucleotide selected from the group consisting of the genes listed in table 3, 4, 5, 6, 7 or 8;

b) detecting the biological activity of the polypeptide of step (a); and

c) selecting a compound that suppresses the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of the genes listed in table 3, 5 and 7 in comparison with the biological activity detected in the absence of the test compound, or enhances the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of the genes listed in table 4, 6 and 8 in comparison with the biological activity detected in the absence of the test compound.

A protein required for the screening can be obtained as a recombinant protein using the nucleotide sequence of the marker gene. Based on the information of the marker gene, one skilled in the art can select any biological activity of the protein as an index for screening and a measurement method based on the selected biological activity.

Alternatively, the screening method of the present invention may comprise the following steps:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of one or more marker genes and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced, wherein the one or more marker genes are selected from the group consisting of the genes listed in table 3, 4, 5, 6, 7 or 8

b) measuring the activity of said reporter gene; and

c) selecting a compound that reduces the expression level of said reporter gene when said marker gene is an up-regulated marker gene selected from the group consisting of the genes listed in table 3, 5 and 7, or that enhances the expression level of said reporter gene when said marker gene is a down-regulated marker gene selected from the group consisting of the genes listed in table 4, 6 and 8, as compared to a control

Suitable reporter genes and host cells are well known in the art. The reporter construct required for the screening can be prepared by using the transcriptional regulatory region of a marker gene. When the transcriptional regulatory region of a marker gene has been known to those skilled in the art, a reporter construct can be prepared by using the previous sequence information. When the transcriptional regulatory region of a marker gene remains unidentified, a nucleotide segment containing the transcriptional regulatory region can be isolated from a genome library based on the nucleotide sequence information of the marker gene.

The compound isolated by the screening is a candidate for drugs that inhibit the activity of the protein encoded by marker genes and can be applied to the treatment or prevention of breast cancer.

Moreover, compound in which a part of the structure of the compound inhibiting the activity of proteins encoded by marker genes is converted by addition, deletion and/or replacement are also included in the compounds obtainable by the screening method of the present invention.

When administrating the compound isolated by the method of the invention as a pharmaceutical for humans and other mammals, such as mice, rats, guinea-pigs, rabbits, cats, dogs, sheep, pigs, cattle, monkeys, baboons, and chimpanzees, the isolated compound can be

directly administered or can be formulated into a dosage form using known pharmaceutical preparation methods. For example, according to the need, the drugs can be taken orally, as sugar-coated tablets, capsules, elixirs and microcapsules, or non-orally, in the form of injections of sterile solutions or suspensions with water or any other pharmaceutically acceptable liquid. For example, the compounds can be mixed with pharmaceutically acceptable carriers or media, specifically, sterilized water, physiological saline, plant-oils, emulsifiers, suspending agents, surfactants, stabilizers, flavoring agents, excipients, vehicles, preservatives, binders, and such, in a unit dose form required for generally accepted drug implementation. The amount of active ingredients in these preparations makes a suitable dosage within the indicated range acquirable.

Examples of additives that can be mixed to tablets and capsules are, binders such as gelatin, corn starch, tragacanth gum and arabic gum; excipients such as crystalline cellulose; swelling agents such as corn starch, gelatin and alginic acid; lubricants such as magnesium stearate; sweeteners such as sucrose, lactose or saccharin; and flavoring agents such as peppermint, Gaultheria adenothrix oil and cherry. When the unit-dose form is a capsule, a liquid carrier, such as an oil, can also be further included in the above ingredients. Sterile composites for injections can be formulated following normal drug implementations using vehicles such as distilled water used for injections.

Physiological saline, glucose, and other isotonic liquids including adjuvants, such as D-sorbitol, D-mannnose, D-mannitol, and sodium chloride, can be used as aqueous solutions for injections. These can be used in conjunction with suitable solubilizers, such as alcohol, specifically ethanol, polyalcohols such as propylene glycol and polyethylene glycol, non-ionic surfactants, such as Polysorbate 80 (TM) and HCO-50.

Sesame oil or Soy-bean oil can be used as a oleaginous liquid and may be used in conjunction with benzyl benzoate or benzyl alcohol as a solubilizer and may be formulated with a buffer, such as phosphate buffer and sodium acetate buffer; a pain-killer, such as procaine hydrochloride; a stabilizer, such as benzyl alcohol andphenol; and an anti-oxidant. The prepared injection may be filled into a suitable ampule.

Methods well known to one skilled in the art may be used to administer the pharmaceutical composition of the present inevntion to patients, for example as intraarterial, intravenous, or percutaneous injections and also as intranasal, transbronchial, intramuscular or oral administrations. The dosage and method of administration vary according to the body-

weight and age of a patient and the administration method; however, one skilled in the art can routinely select a suitable metod of administration. If said compound is encodable by a DNA, the DNA can be inserted into a vector for gene therapy and the vector administered to a patient to perform the therapy. The dosage and method of administration vary according to the body-weight, age, and symptoms of the patient but one skilled in the art can suitably select them.

For example, although the dose of a compound that binds to the protein of the present invention and regulates its activity depends on the symptoms, the dose is about 0.1 mg to about 100 mg per day, preferably about 1.0 mg to about 50 mg per day and more preferably about 1.0 mg to about 20 mg per day, when administered orally to a normal adult (weight 60 kg).

When administering parenterally, in the form of an injection to a normal adult (weight 60 kg), although there are some differences according to the patient, target organ, symptoms and method of administration, it is convenient to intravenously inject a dose of about 0.01 mg to about 30 mg per day, preferably about 0.1 to about 20 mg per day and more preferably about 0.1 to about 10 mg per day. Also, in the case of other animals too, it is possible to administer an amount converted to 60 kgs of body-weight.

Screening assays for identifying therapeutic agents for metastasis of breast cancer

The present invention provides target molecules for treating or preventing metastasis of breast cancer. Screening assay for metastasis of BRC of the present invention can be performed according to the method for BRC described above using marker genes for metastasis of BRC.

In the present invention, marker genes selected from the group consisting of genes listed in table 11 are useful for the screening. 34 genes shown in the Table are associated with lymph node metastasis. Among the genes, 25 genes (+) were relatively up-regulated and 9 genes (-) were down-regulated in node-positive tumors (Table 11 and Figure 7). An agent suppressing the activity or expression of these up-regulated genes obtained by the present invention are useful for treating or preventing BRC with lymph-node metastasis. Alternatively, an agent enhancing the activity or expression of the down-regulated genes obtained by the present invention are also useful for treating or preventing BRC with lymph-node metastasis.

Assessing the prognosis of a subject with breast cancer

Also provided is a method of assessing the prognosis of a subject with BRC by comparing the expression of one or more BRC-associated gene in a test cell population to the expression of the genes in a reference cell population derived from patients over a spectrum of disease stages. By comparing gene expression of one or more BRC-associated gene in the test cell population and the reference cell population(s), or by comparing the pattern of gene expression over time in test cell populations derived from the subject, the prognosis of the subject can be assessed.

A increase in expression of one or more of BRC-associated genes listed in table 3, 5 or 7 compared to a normal control or an decrease of expression of one or more of BRC-associated genes listed in table 4, 6 or 8 compared to a normal control indicates less favorable prognosis. A similar expression of one or more of BRC-associated genes listed in tables 3-8 indicates a more favorable prognosis compared to nomal control indicates a more favorable prognosis for the subject. Preferably, the prognosis of a subject can be assessed by comparing the expression profile of gene selected form group consisting of genes listed in table 3, 4, 5, 6, 7 and 8. The classification score (CS) may be use for the comparing the expression profile.

Kits

The invention also includes a BRC-detection reagent, *e.g.*, a nucleic acid that specifically binds to or identifies one or more BRC nucleic acids such as oligonucleotide sequences, which are complementary to a portion of a BRC nucleic acid or antibodies which bind to proteins encoded by a BRC nucleic acid. The reagents are packaged together in the form of a kit. The reagents are packaged in separate containers, *e.g.*, a nucleic acid or antibody (either bound to a solid matrix or packaged separately with reagents for binding them to the matrix), a control reagent (positive and/or negative), and/or a detectable label. Instructions (*e.g.*, written, tape, VCR, CD-ROM, etc.) for carrying out the assay are included in the kit. The assay format of the kit is a Northern hybridization or a sandwich ELISA known in the art.

For example, BRC detection reagent is immobilized on a solid matrix such as a porous strip to form at least one BRC detection site. The measurement or detection region of the porous strip may include a plurality of sites containing a nucleic acid. A test strip may also contain sites for negative and/or positive controls. Alternatively, control sites are located on a

separate strip from the test strip. Optionally, the different detection sites may contain different amounts of immobilized nucleic acids, *i.e.*, a higher amount in the first detection site and lesser amounts in subsequent sites. Upon the addition of test sample, the number of sites displaying a detectable signal provides a quantitative indication of the amount of BRC present in the sample. The detection sites may be configured in any suitably detectable shape and are typically in the shape of a bar or dot spanning the width of a teststrip.

Alternatively, the kit contains a nucleic acid substrate array comprising one or more nucleic acids. The nucleic acids on the array specifically identify one or more nucleic acid sequences represented by BRC-associated genes listed in tables 3-8. The expression of 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 40 or 50 or more of the nucleic acids represented by BRC-associated genes listed in tables 3-8 are identified by virtue of the level of binding to an array test strip or chip. The substrate array can be on, *e.g.*, a solid substrate, *e.g.*, a "chip" as described in U.S. Patent No.5,744,305.

Arrays and pluralities

The invention also includes a nucleic acid substrate array comprising one or more nucleic acids. The nucleic acids on the array specifically correspond to one or more nucleic acid sequences represented by BRC-associated genes listed in tables 3-8. The level of expression of 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 40 or 50 or more of the nucleic acids represented by BRC-associated genes listed in tables 3-8 are identified by detecting nucleic acid binding to the array.

The invention also includes an isolated plurality (*i.e.*, a mixture of two or more nucleic acids) of nucleic acids. The nucleic acids are in a liquid phase or a solid phase, *e.g.*, immobilized on a solid support such as a nitrocellulose membrane. The plurality includes one or more of the nucleic acids represented by BRC-associated genes listed in tables 3-8. In various embodiments, the plurality includes 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 40 or 50 or more of the nucleic acids represented by BRC-associated genes listed in tables 3-8.

Methods of inhibiting breast cancer

The invention provides a method for treating or alleviating a symptom of BRC in a subject by decreasing expression or activity of BRC-associated genes listed in tables 3, 5, and 7 or increasing expression or activity of BRC-associated genes listed in tables 4, 6, and 8. Therapeutic compounds are administered prophylactically or therapeutically to subject

suffering from or at risk of (or susceptible to) developing BRC. Such subjects are identified using standard clinical methods or by detecting an aberrant level of expression or activity of BRC-associated genes listed in tables 3-8. Therapeutic agents include inhibitors of cell cycle regulation, cell proliferation, and protein kinase activity.

The therapeutic method includes increasing the expression, or function, or both of one or more gene products of genes whose expression is decreased ("under-expressed genes") in a BRC cell relative to normal cells of the same tissue type from which the BRC cells are derived. In these methods, the subject is treated with an effective amount of a compound, which increases the amount of one or more of the under-expressed genes in the subject. Administration can be systemic or local. Therapeutic compounds include a polypeptide product of an under-expressed gene, or a biologically active fragment thereof a nucleic acid encoding an under-expressed gene and having expression control elements permitting expression in the BRC cells; for example an agent which increases the level of expression of such gene endogenous to the BRC cells (i.e., which up-regulates expression of the under-expressed gene or genes). Administration of such compounds counters the effects of aberrantly under-expressed of the gene or genes in the subject's breast cells and improves the clinical condition of the subject.

The method also includes decreasing the expression, or function, or both, of one or more gene products of genes whose expression is aberrantly increased ("over-expressed gene") in breast cells. Expression is inhibited in any of several ways known in the art. For example, expression is inhibited by administering to the subject a nucleic acid that inhibits, or antagonizes, the expression of the over-expressed gene or genes, *e.g.*, an antisense oligonucleotide or small interfering RNA which disrupts expression of the over-expressed gene or genes.

As noted above, antisense nucleic acids corresponding to the nucleotide sequence of BRC-associated genes listed in tables 3, 5, and 7 can be used to reduce the expression level of the genes. Antisense nucleic acids corresponding to BRC-associated genes listed in tables 3, 5, and 7 that are up-regulated in breast cancer are useful for the treatment of breast cancer. Specifically, the antisense nucleic acids of the present invention may act by binding to the BRC-associated genes listed in tables 3, 5, and 7 or mRNAs corresponding thereto, thereby inhibiting the transcription or translation of the genes, promoting the degradation of the mRNAs, and/or inhibiting the expression of proteins encoded by the BRC-associated genes

listed in tables 3, 5, and 7, finally inhibiting the function of the proteins. The term "antisense nucleic acids" as used herein encompasses both nucleotides that are entirely complementary to the target sequence and those having a mismatch of one or more nucleotides, so long as the antisense nucleic acids can specifically hybridize to the target sequences. For example, the antisense nucleic acids of the present invention include polynucleotides that have a homology of at least 70% or higher, preferably at 80% or higher, more preferably 90% or higher, even more preferably 95% or higher over a span of at least 15 continuous nucleotides. Algorithms known in the art can be used to determine the homology.

The antisense nucleic acid derivatives of the present invention act on cells producing the proteins encoded by marker genes by binding to the DNAs or mRNAs encoding the proteins, inhibiting their transcription or translation, promoting the degradation of the mRNAs, and inhibiting the expression of the proteins, thereby resulting in the inhibition of the protein function.

An antisense nucleic acid derivative of the present invention can be made into an external preparation, such as a liniment or a poultice, by mixing with a suitable base material which is inactive against the derivative.

Also, as needed, the derivatives can be formulated into tablets, powders, granules, capsules, liposome capsules, injections, solutions, nose-drops and freeze-drying agents by adding excipients, isotonic agents, solubilizers, stabilizers, preservatives, pain-killers, and such. These can be prepared by following known methods.

The antisense nucleic acids derivative is given to the patient by directly applying onto the ailing site or by injecting into a blood vessel so that it will reach the site of ailment. An antisense-mounting medium can also be used to increase durability and membrane-permeability. Examples are, liposomes, poly-L-lysine, lipids, cholesterol, lipofectin or derivatives of these.

The dosage of the antisense nucleic acid derivative of the present invention can be adjusted suitably according to the patient's condition and used in desired amounts. For example, a dose range of 0.1 to 100 mg/kg, preferably 0.1 to 50 mg/kg can be administered.

The antisense nucleic acids of the invention inhibit the expression of the protein of the invention and is thereby useful for suppressing the biological activity of a protein of the invention. Also, expression-inhibitors, comprising the antisense nucleic acids of the invention, are useful since they can inhibit the biological activity of a protein of the invention.

The antisense nucleic acids of present invention include modified oligonucleotides. For example, thioated nucleotides may be used to confer nuclease resistance to an oligonucleotide.

Also, a siRNA against marker gene can be used to reduce the expression level of the marker gene. By the term "siRNA" is meant a double stranded RNA molecule which prevents translation of a target mRNA. Standard techniques of introducing siRNA into the cell are used, including those in which DNA is a template from which RNA is transcribed. In the context of the present invention, the siRNA comprises a sense nucleic acid sequence and an anti-sense nucleic acid sequence against an up-regulated marker gene, such as BRC-associated genes listed in tables 3, 5, and 7. The siRNA is constructed such that a single transcript has both the sense and complementary antisense sequences from the target gene, *e.g.*, a hairpin.

The method is used to alter the expression in a cell of an up-regulated, *e.g.*, as a result of malignant transformation of the cells. Binding of the siRNA to a transcript corresponding to one of the BRC-associated genes listed in tables 3, 5, and 7 in the target cell results in a reduction in the protein production by the cell. The length of the oligonucleotide is at least 10 nucleotides and may be as long as the naturally-occurring transcript. Preferably, the oligonucleotide is 19-25 nucleotides in length. Most preferably, the oligonucleotide is less than 75, 50, 25 nucleotides in length.

The nucleotide sequence of the siRNAs were designed using an siRNA design computer program available from the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html). The computer program selects nucleotide sequences for siRNA synthesis based on the following protocol.

Selection of siRNA Target Sites:

1. Beginning with the AUG start codon of the object transcript, scan downstream for AA dinucleotide sequences. Record the occurrence of each AA and the 3' adjacent 19 nucleotides as potential siRNA target sites. Tuschl, et al. recommend against designing siRNA to the 5' and 3' untranslated regions (UTRs) and regions near the start codon (within 75 bases) as these may be richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex.

2. Compare the potential target sites to the human genome database and eliminate from consideration any target sequences with significant homology to other coding sequences. The homology search can be performed using BLAST, which can be found on the NCBI server at: www.ncbi.nlm.nih.gov/BLAST/
3. Select qualifying target sequences for synthesis. At Ambion, preferably several target sequences can be selected along the length of the gene to evaluate.

The antisense oligonucleotide or siRNA of the invention inhibit the expression of the polypeptide of the invention and is thereby useful for suppressing the biological activity of the polypeptide of the invention. Also, expression-inhibitors, comprising the antisense oligonucleotide or siRNA of the invention, are useful in the point that they can inhibit the biological activity of the polypeptide of the invention. Therefore, a composition comprising the antisense oligonucleotide or siRNA of the present invention is useful in treating a breast cancer.

Alternatively, function of one or more gene products of the over-expressed genes is inhibited by administering a compound that binds to or otherwise inhibits the function of the gene products. For example, the compound is an antibody which binds to the over-expressed gene product or gene products.

The present invention refers to the use of antibodies, particularly antibodies against a protein encoded by an up-regulated marker gene, or a fragment of the antibody. As used herein, the term "antibody" refers to an immunoglobulin molecule having a specific structure, that interacts (i.e., binds) only with the antigen that was used for synthesizing the antibody (i.e., the up-regulated marker gene product) or with an antigen closely related to it. Furthermore, an antibody may be a fragment of an antibody or a modified antibody, so long as it binds to one or more of the proteins encoded by the marker genes. For instance, the antibody fragment may be Fab, $F(ab')_2$, Fv, or single chain Fv (scFv), in which Fv fragments from H and L chains are ligated by an appropriate linker (Huston J. S. et al. Proc. Natl. Acad. Sci. U.S.A. 85:5879-5883 (1988)). More specifically, an antibody fragment may be generated by treating an antibody with an enzyme, such as papain or pepsin. Alternatively, a gene encoding the antibody fragment may be constructed, inserted into an expression vector, and expressed in an appropriate host cell (see, for example, Co M. S. et al. J. Immunol. 152:2968-2976 (1994); Better M. and Horwitz A. H. Methods Enzymol. 178:476-496 (1989); Pluckthun A. and Skerra A. Methods Enzymol. 178:497-515 (1989); Lamoyi E. Methods Enzymol.

121:652-663 (1986); Rousseaux J. et al. Methods Enzymol. 121:663-669 (1986); Bird R. E. and Walker B. W. Trends Biotechnol. 9:132-137 (1991)).

An antibody may be modified by conjugation with a variety of molecules, such as polyethylene glycol (PEG). The present invention provides such modified antibodies. The modified antibody can be obtained by chemically modifying an antibody. These modification methods are conventional in the field.

Alternatively, an antibody may be obtained as a chimeric antibody, between a variable region derived from a nonhuman antibody and a constant region derived from a human antibody, or as a humanized antibody, comprising the complementarity determining region (CDR) derived from a nonhuman antibody, the frame work region (FR) derived from a human antibody, and the constant region. Such antibodies can be prepared by using known technologies.

Cancer therapies directed at specific molecular alterations that occur in cancer cells have been validated through clinical development and regulatory approval of anti-cancer drugs such as trastuzumab (Herceptin) for the treatment of advanced breast cancer, imatinib methylate (Gleevec) for chronic myeloid leukemia, gefitinib (Iressa) for non-small cell lung cancer (NSCLC), and rituximab (anti-CD20 mAb) for B-cell lymphoma and mantle cell lymphoma (Ciardiello F, Tortora G. A novel approach in the treatment of cancer: targeting the epidermal growth factor receptor. Clin Cancer Res. 2001 Oct;7(10):2958-70. Review.; Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med. 2001 Mar 15;344(11):783-92.; Rehwald U, Schulz H, Reiser M, Sieber M, Staak JO, Morschhauser F, Driessen C, Rudiger T, Muller-Hermelink K, Diehl V, Engert A. Treatment of relapsed CD20+ Hodgkin lymphoma with the monoclonal antibody rituximab is effective and well tolerated: results of a phase 2 trial of the German Hodgkin Lymphoma Study Group. Blood. 2003 Jan 15;101(2):420-424.; Fang G, Kim CN, Perkins CL, Ramadevi N, Winton E, Wittmann S and Bhalla KN. (2000). Blood, 96, 2246-2253.). These drugs are clinically effective and better tolerated than traditional anti-cancer agents because they target only transformed cells. Hence, such drugs not only improve survival and quality of life for cancer patients, but also validate the concept of molecularly targeted cancer therapy. Furthermore, targeted drugs can enhance the efficacy of standard chemotherapy when used in combination

with it (Gianni L. (2002). Oncology, 63 Suppl 1, 47-56.; Klejman A, Rushen L, Morrione A, Slupianek A and Skorski T. (2002). Oncogene, 21, 5868-5876.). Therefore, future cancer treatments will probably involve combining conventional drugs with target-specific agents aimed at different characteristics of tumor cells such as angiogenesis and invasiveness.

These modulatory methods are performed ex vivo or in vitro (*e.g.*, by culturing the cell with the agent) or, alternatively, in vivo (*e.g.*, by administering the agent to a subject). The method involves administering a protein or combination of proteins or a nucleic acid molecule or combination of nucleic acid, molecules as therapy to counteract aberrant expression or activity of the differentially expressed genes.

Diseases and disorders that are characterized by increased (relative to a subject not suffering from the disease or disorder) levels or biological activity of the genes may be treated with therapeutics that antagonize (i.e., reduce or inhibit) activity of the over-expressed gene or genes. Therapeutics that antagonized activity are administered therapeutically or prophylactically.

Therapeutics that may be utilized include, *e.g.*, (i) a polypeptide, or analogs, derivatives, fragments or homologs thereof of the over-expressed or under-expressed gene or genes; (ii) antibodies to the over-expressed gene or genes; (iii) nucleic acids encoding the over-expressed or under-expressed gene or genes; (iv) antisense nucleic acids or nucleic acids that are "dysfunctional" (i.e., due to a heterologous insertion within the nucleic acids of one or more over-expressed gene or genes); (v) small interfering RNA (siRNA); or (vi) modulators (i.e., inhibitors, agonists and antagonists that alter the interaction between an over-expressed polypeptide and its binding partner). The dysfunctional antisense molecules are utilized to "knockout" endogenous function of a polypeptide by homologous recombination (see, *e.g.*, Capecchi, Science 244: 1288-1292 1989).

Diseases and disorders that are characterized by decreased (relative to a subject not suffering from the disease or disorder) biological activity may be treated with therapeutics that increase (i.e., are agonists to) activity. Therapeutics that up-regulate activity may be administered in a therapeutic or prophylactic manner. Therapeutics that may be utilized include, but are not limited to, a polypeptide (or analogs, derivatives, fragments or homologs thereof) or an agonist that increases bioavailability.

Increased or decreased levels can be readily detected by quantifying peptide and/or RNA, by obtaining a patient tissue sample (*e.g.*, from biopsy tissue) and assaying it in vitro

for RNA or peptide levels, structure and/or activity of the expressed peptides (or mRNAs of a gene whose expression is altered). Methods that are well-known within the art include, but are not limited to, immunoassays (*e.g.*, by Western blot analysis, immunoprecipitation followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect expression of mRNAs (*e.g.*, Northern assays, dot blots, in situ hybridization, etc.).

Prophylactic administration occurs prior to the manifestation of overt clinical symptoms of disease, such that a disease or disorder is prevented or, alternatively, delayed in its progression.

Therapeutic methods include contacting a cell with an agent that modulates one or more of the activities of the gene products of the differentially expressed genes. An agent that modulates protein activity includes a nucleic acid or a protein, a naturally-occurring cognate ligand of these proteins, a peptide, a peptidomimetic, or other small molecule. For example, the agent stimulates one or more protein activities of one or more of a differentially under-expressed gene.

The present invention also relates to a method of treating or preventing breast cancer in a subject comprising administering to said subject a vaccine comprising a polypeptide encoded by a nucleic acid selected from the group consisting of BRC-associated genes listed in tables 3, 5, and 7 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide or the fragment thereof. An administration of the polypeptide induces an anti-tumor immunity in a subject. To inducing anti-tumor immunity, a polypeptide encoded by a nucleic acid selected from the group consisting of BRC-associated genes listed in tables 3, 5, and 7 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide is administered. The polypeptide or the immunologically active fragments thereof are useful as vaccines against BRC. In some cases the proteins or fragments thereof may be administered in a form bound to the T cell recepor (TCR) or presented by an antigen presenting cell (APC), such as macrophage, dendritic cell (DC), or B-cells. Due to the strong antigen presenting ability of DC, the use of DC is most preferable among the APCs.

In the present invention, vaccine against BRC refers to a substance that has the function to induce anti-tumor immunity upon inoculation into animals. According to the present invention, polypeptides encoded by BRC-associated genes listed in tables 3, 5, and 7

or fragments thereof were suggested to be HLA-A24 or HLA-A*0201 restricted epitopes peptides that may induce potent and specific immune response against BRC cells expressing BRC-associated genes listed in tables 3, 5, and 7. Thus, the present invention also encompasses method of inducing anti-tumor immunity using the polypeptides. In general, anti-tumor immunity includes immune responses such as follows:

- induction of cytotoxic lymphocytes against tumors,
- induction of antibodies that recognize tumors, and
- induction of anti-tumor cytokine production.

Therefore, when a certain protein induces any one of these immune responses upon inoculation into an animal, the protein is decided to have anti-tumor immunity inducing effect. The induction of the anti-tumor immunity by a protein can be detected by observing in vivo or in vitro the response of the immune system in the host against the protein.

For example, a method for detecting the induction of cytotoxic T lymphocytes is well known. A foreign substance that enters the living body is presented to T cells and B cells by the action of antigen presenting cells (APCs). T cells that respond to the antigen presented by APC in antigen specific manner differentiate into cytotoxic T cells (or cytotoxic T lymphocytes; CTLs) due to stimulation by the antigen, and then proliferate (this is referred to as activation of T cells). Therefore, CTL induction by a certain peptide can be evaluated by presenting the peptide to T cell by APC, and detecting the induction of CTL. Furthermore, APC has the effect of activating CD4+ T cells, CD8+ T cells, macrophages, eosinophils, and NK cells. Since CD4+ T cells and CD8+ T cells are also important in anti-tumor immunity, the anti-tumor immunity inducing action of the peptide can be evaluated using the activation effect of these cells as indicators.

A method for evaluating the inducing action of CTL using dendritic cells (DCs) as APC is well known in the art. DC is a representative APC having the strongest CTL inducing action among APCs. In this method, the test polypeptide is initially contacted with DC, and then this DC is contacted with T cells. Detection of T cells having cytotoxic effects against the cells of interest after the contact with DC shows that the test polypeptide has an activity of inducing the cytotoxic T cells. Activity of CTL against tumors can be detected, for example, using the lysis of ^{51}Cr-labeled tumor cells as the indicator. Alternatively, the method of evaluating the degree of tumor cell damage using ^{3}H-thymidine uptake activity or LDH (lactose dehydrogenase)-release as the indicator is also well known.

Apart from DC, peripheral blood mononuclear cells (PBMCs) may also be used as the APC. The induction of CTL is reported that it can be enhanced by culturing PBMC in the presence of GM-CSF and IL-4. Similarly, CTL has been shown to be induced by culturing PBMC in the presence of keyhole limpet hemocyanin (KLH) and IL-7.

The test polypeptides confirmed to possess CTL inducing activity by these methods are polypeptides having DC activation effect and subsequent CTL inducing activity. Therefore, polypeptides that induce CTL against tumor cells are useful as vaccines against tumors. Furthermore, APC that acquired the ability to induce CTL against tumors by contacting with the polypeptides are useful as vaccines against tumors. Furthermore, CTL that acquired cytotoxicity due to presentation of the polypeptide antigens by APC can be also used as vaccines against tumors. Such therapeutic methods for tumors using anti-tumor immunity due to APC and CTL are referred to as cellular immunotherapy.

Generally, when using a polypeptide for cellular immunotherapy, efficiency of the CTL-induction is known to increase by combining a plurality of polypeptides having different structures and contacting them with DC. Therefore, when stimulating DC with protein fragments, it is advantageous to use a mixture of multiple types of fragments.

Alternatively, the induction of anti-tumor immunity by a polypeptide can be confirmed by observing the induction of antibody production against tumors. For example, when antibodies against a polypeptide are induced in a laboratory animal immunized with the polypeptide, and when growth of tumor cells is suppressed by those antibodies, the polypeptide can be determined to have an ability to induce anti-tumor immunity.

Anti-tumor immunity is induced by administering the vaccine of this invention, and the induction of anti-tumor immunity enables treatment and prevention of BRC. Therapy against cancer or prevention of the onset of cancer includes any of the steps, such as inhibition of the growth of cancerous cells, involution of cancer, and suppression of occurrence of cancer. Decrease in mortality of individuals having cancer, decrease of tumor markers in the blood, alleviation of detectable symptoms accompanying cancer, and such are also included in the therapy or prevention of cancer. Such therapeutic and preventive effects are preferably statistically significant. For example, in observation, at a significance level of 5% or less, wherein the therapeutic or preventive effect of a vaccine against cell proliferative diseases is compared to a control without vaccine administration. For example, Student's t-test, the Mann-Whitney U-test, or ANOVA may be used for statistical analysis.

The above-mentioned protein having immunological activity or a vector encoding the protein may be combined with an adjuvant. An adjuvant refers to a compound that enhances the immune response against the protein when administered together (or successively) with the protein having immunological activity. Examples of adjuvants include cholera toxin, salmonella toxin, alum, and such, but are not limited thereto. Furthermore, the vaccine of this invention may be combined appropriately with a pharmaceutically acceptable carrier. Examples of such carriers are sterilized water, physiological saline, phosphate buffer, culture fluid, and such. Furthermore, the vaccine may contain as necessary, stabilizers, suspensions, preservatives, surfactants, and such. The vaccine is administered systemically or locally. Vaccine administration may be performed by single administration, or boosted by multiple administrations.

When using APC or CTL as the vaccine of this invention, tumors can be treated or prevented, for example, by the ex vivo method. More specifically, PBMCs of the subject receiving treatment or prevention are collected, the cells are contacted with the polypeptide ex vivo, and following the induction of APC or CTL, the cells may be administered to the subject. APC can be also induced by introducing a vector encoding the polypeptide into PBMCs ex vivo. APC or CTL induced in vitro can be cloned prior to administration. By cloning and growing cells having high activity of damaging target cells, cellular immunotherapy can be performed more effectively. Furthermore, APC and CTL isolated in this manner may be used for cellular immunotherapy not only against individuals from whom the cells are derived, but also against similar types of tumors from other individuals.

Furthermore, a pharmaceutical composition for treating or preventing a cell proliferative disease, such as cancer, comprising a pharmaceutically effective amount of the polypeptide of the present invention is provided. The pharmaceutical composition may be used for raising anti tumor immunity.

Pharmaceutical compositions for inhibiting BRC or malignant BRC.

Pharmaceutical formulations include those suitable for oral, rectal, nasal, topical (including buccal and sub-lingual), vaginal or parenteral (including intramuscular, sub-cutaneous and intravenous) administration, or for administration by inhalation or insufflation. Preferably, administration is intravenous. The formulations are optionally packaged in discrete dosage units.

Pharmaceutical formulations suitable for oral administration include capsules, cachets or tablets, each containing a predetermined amount of the active ingredient. Formulations also include powders, granules or solutions, suspensions or emulsions. The active ingredient is optionally administered as a bolus electuary or paste. Tablets and capsules for oral administration may contain conventional excipients such as binding agents, fillers, lubricants, disintegrant or wetting agents. A tablet may be made by compression or molding, optionally with one or more formulational ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredients in a free-flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, lubricating, surface active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may be coated according to methods well known in the art. Oral fluid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which may include edible oils), or preservatives. The tablets may optionally be formulated so as to provide slow or controlled release of the active ingredient therein. A package of tablets may contain one tablet to be taken on each of the month.

Formulations for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline, water-for-injection, immediately prior to use. Alternatively, the formulations may be presented for continuous infusion. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Formulations for rectal administration include suppositories with standard carriers such as cocoa butter or polyethylene glycol. Formulations for topical administration in the mouth, for example buccally or sublingually, include lozenges, which contain the active

ingredient in a flavored base such as sucrose and acacia or tragacanth, and pastilles comprising the active ingredient in a base such as gelatin and glycerin or sucrose and acacia. For intra-nasal administration the compounds of the invention may be used as a liquid spray or dispersible powder or in the form of drops. Drops may be formulated with an aqueous or non-aqueous base also comprising one or more dispersing agents, solubilizing agents or suspending agents.

For administration by inhalation the compounds are conveniently delivered from an insufflator, nebulizer, pressurized packs or other convenient means of delivering an aerosol spray. Pressurized packs may comprise a suitable propellant such as dichlorodifluoromethane, trichlorofluoromethane, dichiorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount.

Alternatively, for administration by inhalation or insufflation, the compounds may take the form of a dry powder composition, for example a powder mix of the compound and a suitable powder base such as lactose or starch. The powder composition may be presented in unit dosage form, in for example, capsules, cartridges, gelatin or blister packs from which the powder may be administered with the aid of an inhalator or insufflators.

Other formulations include implantable devices and adhesive patches; which release a therapeutic agent.

When desired, the above described formulations, adapted to give sustained release of the active ingredient, may be employed. The pharmaceutical compositions may also contain other active ingredients such as antimicrobial agents, immunosuppressants or preservatives.

It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable for oral administration may include flavoring agents.

Preferred unit dosage formulations are those containing an effective dose, as recited below, or an appropriate fraction thereof, of the active ingredient.

For each of the aforementioned conditions, the compositions, *e.g.*, polypeptides and organic compounds are administered orally or via injection at a dose of from about 0.1 to about 250 mg/kg per day. The dose range for adult humans is generally from about 5 mg to about 17.5 g/day, preferably about 5 mg to about 10 g/day, and most preferably about 100 mg

to about 3 g/day. Tablets or other unit dosage forms of presentation provided in discrete units may conveniently contain an amount which is effective at such dosage or as a multiple of the same, for instance, units containing about 5 mg to about 500 mg, usually from about 100 mg to about 500 mg.

The dose employed will depend upon a number of factors, including the age and sex of the subject, the precise disorder being treated, and its severity. Also the route of administration may vary depending upon the condition and its severity.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims. The following examples illustrate the identification and characterization of genes differentially expressed in BRC cells.

EXAMPLE

Tissue obtained from diseased tissue (*e.g.*, epithelial cells from BRC) and normal tissues was evaluated to identify genes which are differently expressed or a disease state, *e.g.*, BRC. The assays were carried out as follows.

Patients, tissue samples

Primary breast cancers were obtained with informed consent from 81 patients (12 ductal carcinoma in situ and 69 invasive ductal carcinoma from 2 cm to 5 cm(T2), median age 45 in a range of 21 to 68 years old) who treated at Department of Breast Surgery, Cancer Institute Hospital, Tokyo, Japan, concerning which all patients had given informed consent (Table 12). Clinical information was obtained from medical records and each tumor was diagnosed according to histopathological subtype and grade by pathologists. Tumor tissue was used to evaluate tumor type (according to the World Health Organization classification and the Japanese cancer society classification). Clinical stage was judged according to the JBCS TNM classification. No significant differences were observed between node-positive and node-negative cases. The presence of angioinvasive growth and extensive lymphocytic infiltrate was determined by pathologists, Estrogen receptor (ER) and progesterone receptor (PgR) expression was determined by EIA (ER negative when less than 13fmol/mg protein, BML). A mixture of normal breast ductal cells from the 15 premenopausal patients with breast cancer or the 12 post menopausal patients were used as normal controls, respectively.

All samples were immediately frozen and stored at -80°C.

Tissue Samples and LMM

Clinical and pathological information on the tumor is detailed in Table 13. Samples were embedded in TissueTek OCT medium (Sakura) and then stored at -80°C until use. Frozen specimens were serially sectioned in 8-μm slices with a cryostat and stained with hematoxylin and eosin to define the analyzed regions. To avoid cross-contamination of cancer and noncancerous cells, we prepared these two populations by EZ Cut LMM System (SL Microtest GmbH) followed the manufacture's protocol with several modifications. To minimize the effects during storage process and tissue collection, we carefully handled the cancer tissues by the same procedure. To check the quality of RNAs, total RNA extracted from the residual tissue of each case were electrophoresed under the degenerative agarose gel, and confirmed their quality by a presence of ribosomal RNA bands.

RNA Extraction and T7-Based RNA Amplification

Total RNA was extracted from each population of laser captured cells into 350μl RLT lysis buffer (QIAGEN). The extracted RNA was treated for 30 minutes at room temperature with 30 units of DNase I (QIAGEN). After inactivation at 70°C for 10 min, the RNAs were purified with an RNeasy Mini Kit (QIAGEN) according to the manufacturer's recommendations. All of the DNase I treated RNA was subjected to T7-based amplification using Ampliscribe T7 Transcription Kit (Epicentre Technologies). Two rounds of amplification yielded 28.8-329.4 μg of amplified RNAs (aRNAs) for each sample, whereas when we amplified RNAs from normal samples from 15 premenopausal patients or 12 postmenopausal patients, total of 2240.2μg and 2023.8μg were yielded, respectively. 2.5μg aliquots of aRNA from each cancerous cells and noncancerous breast ductal cells were reverse-transcribed in the presence of Cy5-dCTP and Cy3-dCTP (Amersham Biosciences), respectively.

cDNA microarrays

We established a "genome-wide" cDNA microarray system containing 23,040 cDNAs selected from the UniGene database (build #131) the National Center for Biotechnology Information (NCBI). Briefly, the cDNAs were amplified by reverse

transcription-PCR using poly(A)+RNA isolated from various human organs as templates; lengths of the amplicons ranged from 200 to 1100 bp without repetitive or poly(A) sequences. The PCR products were spotted in duplicate on type-7 glass slides (Amersham Bioscience) using a Lucidea Array Spotter (Amersham Biosciences); 4,608 or 9,216 genes were spotted in duplicate on a single slide. We prepared three different sets of slides (total 23,040 genes), on each of which the same 52 housekeeping genes and two kinds of negative-control genes were spotted as well.

Hybridization and Acquisition of Data

Hybridization and washing were performed according to protocols described previously except that all processes were carried out with an Automated Slide Processor (Amersham Biosciences) (Giuliani, N., et al., V. Human myeloma cells stimulate the receptor activator of nuclear factor-kappa B ligand (RANKL) in T lymphocytes: a potential role in multiple myeloma bone disease. Blood, *100:* 4615-4621, 2002.). The intensity of each hybridization signal was calculated photometrically by the ArrayVision computer program (Amersham Biosciences) and background intensity was subtracted. The fluorescence intensities of Cy5 (tumor) and Cy3 (control) for each target spot were adjusted so the mean Cy5/Cy3 ratio was performed using averaged signals from the 52 housekeeping genes. Because data derived from low signal intensities are less reliable, we determined a cut-off value for signal intensities on each slide and excluded genes from further analysis when both Cy3 and Cy5 dyes gave signal intensities lower than the cut-off. A cut-off value for each expression level was automatically calculated according to background fluctuation. When both Cy5 and Cy3 signal intensities were lower than the cut-off values, we assessed expression of the corresponding gene in that sample as absent. We calculated Cy5/Cy3 as the relative expression ratio. For other genes we calculated the Cy5/Cy3 ratio using raw data of each sample.

Signal intensities of Cy3 and Cy5 from the 23,040 spots were quantified and analyzed by substituting backgrounds, using ArrayVision software (Imaging Research, Inc., St. Catharines, Ontario, Canada). Subsequently the fluorescent intensities of Cy5 (tumor) and Cy3 (control) for each target spot were adjusted so that the mean Cy3/Cy5 ratio of 52 housekeeping genes on the array was equal to one. Because data derived from low signal intensities are less reliable, we determined a cut-off value on each slide as described

previously (Ono, K., et al.,Identification by cDNA microarray of genes involved in ovarian carcinogenesis. Cancer Res, *60:* 5007-5011, 2000.) excluded genes from further analysis when both Cy3 and Cy5 dyes yielded signal intensities lower than the cut-off (Saito-Hisaminato, A., Katagiri, T., Kakiuchi, S., Nakamura, T., Tsunoda, T., and Nakamura, Y. Genome-wide profiling of gene expression in 29 normal human tissues with a cDNA microarray. DNA Res, *9:* 35-45, 2002.). For other genes we calculated the Cy5/Cy3 ratio using the raw data of each sample.

Calculation of Contamination Percentage

Perilipin (*PLIN*), fatty acid binding protein 4 (*FABP4*) were expressed exclusively in adipose tissue and mammaly gland tissue by gene expression profiles in 29 normal human tissues with a cDNA microarray (Saito-Hisaminato, A. et al., Genome-wide profiling of gene expression in 29 normal human tissues with a cDNA microarray. DNA Res, *9:* 35-45, 2002.). These were used to evaluate the proportion of adipocytes present in the population of microdissected normal breast ductal epithelial cells. Each aRNA of poly A^+RNA isolated from normal whole-mammary gland (Clontech) and of microdissected normal breast ductal epithelial cells were reverse-transcribed in the presence of Cy5-dCTP and Cy3-dCTP, respectively. After hybridization on microarray slides, we calculated Cy5/Cy3 ratio. The average of each ratio was decided by the result used mammary gland tissue and microdissected normal breast ductal cells in premenopausal patients and postmenopausal patients.

Cluster analysis of 102 samples with 81 breast carcinoma according to gene-expression profiles

We applied unsupervised hierarchical hierarchical clustering method to both genes and tumors. To obtain reproducible clusters for classification of the 102 samples, we selected 710 genes for which valid data were obtained in 80% of the experiments, and whose expression ratios varied by standard deviations of more than 1.1. The analysis was performed using web-available software ("Cluster" and "TreeView") written by M. Eisen (http://genome-www5.stanford.edu/ MicroArray/SMD/restech.html). Before applying the clustering algorithm, we log-transformed the fluorescence ratio for each spot and then median-centered the data for each sample to remove experimental biases and used average linkage.

Identification of Up or down-Regulated Genes between DCIS and IDC.

The relative expression ratio of each gene (Cy5/Cy3 intensity ratio) was classified into one of four categories: (A) up-regulated (expression ratio >2.0); (B) down-regulated (expression ratio <0.5); (C) unchanged (expression ratio between 0.5 and 2.0); and (D) not expressed (or slight expression but under the cutoff level for detection). We used these categories to detect a set of genes for which changes in the expression ratios were common among samples. To detect candidate genes that were commonly up or down-regulated in each group, the overall expression patterns of 23,040 genes were first screened to select genes with expression ratios >3.0 or <1/3 that were >50% in present in >50% of the groups categorized.

Semi-quantitative RT-PCR

We selected the 5 up-regulated genes and examined their expression levels by applying the semi-quantitative RT-PCR experiments. A 1-μg aliquot of aRNA from each sample was reverse-transcribed for single-stranded cDNAs using random primer (Taniguchi, K., et al., Mutational spectrum of beta-catenin, AXIN1, and AXIN2 in hepatocellular carcinomas and hepatoblastomas. Oncogene, *21:* 4863-4871, 2002.) and Superscript II (Life Technologies, Inc.). Each cDNA mixture was diluted for subsequent PCR amplification with the primer sets that were shown in Table 9. Expression of GAPDH served as an internal control. PCR reactions were optimized for the number of cycles to ensure product intensity within the linear phase of amplification.

Identification of genes responsible for Histopathological status, ER status and lymph-node metastasis in breast cancer

We selected the discriminating genes using the following two criteria; (1) signal intensities higher than the cut-off level in at least 70% (ER status) 50% (Histopathological status and lymph-node metastasis of the cases); (2) $|Med_r - Med_n| > 1$ (ER status) or 0.5 (Histopathological status and lymph-node metastasis of the cases), where Med indicates the median derived from log-transformed relative expression ratios in node-positive cases or - negative cases. Next, we applied a random permutation test to identify genes that were expressed differently between one group (group A) and another (group B). Mean (μ) and

standard (σ) deviations were calculated from the log-transformed relative expression ratios of each gene in group A (r) and group B (n) cases. A discrimination score (DS) for each gene was defined as follows:

$$DS = (\mu_r - \mu_n) / (\sigma_r + \sigma_n)$$

We carried out permutation tests to estimate the ability of individual genes to distinguish between group A and group B; samples were randomly permutated between the two classes 10,000 times. Since the DS dataset of each gene showed a normal distribution, we calculated a P value for the user-defined grouping (Golub, T. et al., Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science, *286:* 531-537, 1999.).

Calculation of prediction score for lymph-node metastasis

We calculated prediction scores according to procedures described previously (Golub, T. et al., Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science, *286:* 531-537, 1999.). Each gene (gi) votes for either lymph node-negative or lymph node-positive depending on whether the expression level (xi) in the sample is closer to the mean expression level of node-negative or -positive in reference samples. The magnitude of the vote (vi) reflects the deviation of the expression level in the sample from the average of the two classes:

$$V_i = | x_i - (\mu_r + \mu_n) /2 |$$

We summed the votes to obtain total votes for the node-negative (Vr) and node-positive (Vn), and calculated PS values as follows:

PS = (Vr – Vn) / (Vr + Vn) x 100, reflecting the margin of victory in the direction of either node-negative or node-positive. PS values range from –100 to 100; a higher absolute value of PS reflects a stronger prediction.

Evaluation of classification and leave-one-out test

We calculated the classification score (CS) using prediction scores of lymph node-negatives (PSr) and node-positives (PSn) in each gene set, as follows:

$$CS = (\mu_{PSr} - \mu_{PSn}) / (\sigma_{PSr} + \sigma_{PSn})$$

A larger value of CS indicates better separation of the two groups by the predictive-scoring

system. For the leave-one-out test, one sample is withheld, the permutation p-value and mean expression levels are calculated using remaining samples, and the class of the withheld sample is subsequently evaluated by calculating its prediction score. We repeated this procedure for each of the 20 samples.

RESULTS

Classification analysis on the basis of precise gene expression profiles of breast cancer

Since breast cancer contains a low population of cancer cells in tumor mass and originates from normal epithelial duct cells, we carried out microdissection to avoid contamination of the surrounding non-cancerous cells or non-normal ductal epithelial cells. As the great majority of cells in breast tissue are adipocytes, we considered it is not suitable to use the whole breast tissue to analyze cancer-specific expression profiles in that organ. As shown in Figure 1, the representative examples of DCIS (case 10326T), IDC (10502T), and normal ductal epithelium (10341N) were microdissected from each clinical specimen. This allows us the subsequent gene expression profiles to be obtained more preciously. We also examined the proportion of adipocytes that were contaminating the microdissected population of normal breast ductal epithelial cells serving as a universal control, by measuring the signal intensities of genes (*PLIN and FABP4*) that are highly expressed in adipose and mammary gland tissues as described previously (Saito-Hisaminato, A., et al.,. Genome-wide profiling of gene expression in 29 normal human tissues with a cDNA microarray. DNA Res, *9:* 35-45, 2002.). When we investigated the signal intensities of these genes in whole mammary gland tissue which contains a large number of adipocytes, the average of ratio of signal intensities of these gene were approximately 99.4 %; the ratio in microdissected normal breast ductal epithelial cells was approximately 0.6 % (see Contamination percentage section in Materials and Methods). Therefore, we estimated the average proportion of contaminating adipocytes in the populations of control cells to be 0.6% after microdissection.

We first applied unsupervised two-dimensional hierarchical clustering algorithm to group genes on the basis of similarity in their expression pattern over 102 clinical samples: 81 microdissected different clinical breast cancer specimens, 11 microdissected different histological types in 10 individuals, 2 whole breast cancer tissues, 6 microdissected normal breast ductal cells and two whole mammary gland tissues. Reproducible clusters were

obtained with 710 genes (see Material and methods); their expression patterns across the 102 samples are shown in Figure 2A. In the sample axis, the 102 samples were clustered into three major groups (Group A, B and C) on the basis of their expression profiles. Then we associated this classification with clinical parameters, especially estrogen receptor (ER) as determined with EIA. Out of 55 ER-positive tumors, 45 cases clustered into same branch (Group B) of the tumor dendrogram, suggesting a tendency with ER status. Moreover, 7 of 10 cases with different histological type (sample# 10864, 10149, 10818, 10138, 10005, 10646 and 10435) was labeled and hybridized in independent experiment were clustered most closely within same group. In particular, among them, the one duplicated case (10149a1 and 10149a1T) was also clustered into the shortest branch, supporting the reproducibility and reliability of our microarray data. Remarkably, Group C contained microdissected non-cancerous cells and breast cancer whole tissues except one microdissected tumor case, suggesting this data represents accurate breast cancer specific-expression profiles.

Furthermore, we performed two-dimensional hierarchical clustering analysis of 89 genes across 16 samples with 2 differentiated lesion microdissected from 8 breast cancer patients. As a result, breast cancer samples with different phenotype lesions was closely adjacent (Figure 2B). We next carried out random permutation test to identify the genes that were differentially expressed in the patient-matched phenotypically well- or poorly-differentiated lesions from microdissected 8 cancer specimen. As shown in Figure 2C, clustering analysis using 25 genes that showed differentially expression can separate between well- or poorly-differentiated invasive ductal cancer cells. These 25 genes (Table 1) included some key factors whose possible roles in invasion and cell growth had been reported previously: *TNFSF11*, *ITGA5* and *NFAT5* (Giuliani, N., et al., Human myeloma cells stimulate the receptor activator of nuclear factor-kappa B ligand (RANKL) in T lymphocytes: a potential role in multiple myeloma bone disease. Blood, *100:* 4615-4621, 2002.; Sebastien J. et al., The role of NFAT transcription factors in integrin-mediated carcinoma invasion. Nature cell biology, *4:* 540-544, 2002.; Klein, S. et al., Alpha 5 beta 1 integrin activates an NF-kappa B-dependent program of gene expression important for angiogenesis and inflammation. Mol Cell Biol, *22:* 5912-5922, 2002.).

Next, we carried out random permutation test to identify the genes that were differentially expressed in 41 ER-positive tumor and 28 ER-negative tumors in IDC. These

all samples were premenopausal patients. We listed 97 genes that were able to distinguish between ER positive and negative with permutation P-value of less than 0.0001 (see "Materials and Methods") (Figure 3 and Table2). Expression levels were increased for 92 of those genes and decreased for the other five in ER-positive group, as compared to the ER-negative group. Among these genes, GATA binding protein 3 (*GATA3*), trefoil factor 3 (*TFF3*), cyclin D1 (*CCND1*), MAPKK homolog (*MAP2K4*) and tissue inhibitor of metalloprotease 1 (*TIMP1*), insulin receptor substrate 1(*IRS1*), X-box binding protein 1(*XBP1*), GLI-Kruppel family memberGLI3(*GLI3*) were over-expressed in the ER-positives (Table 2). In addition, since estrogen receptor (ESR1) was rank-ordered at 6th gene on the basis of magnitude of p-value (bottom panel in Figure 3), it might be possible to distinguish breast cancers according to expression profiles of ER.

Identification of commonly up- or down-regulated genes in DCIS or IDC

To further clarify mechanisms underlying carcinogenesis of breast cancer, we initially searched for genes commonly up- or down-regulated in DCIS and IDC, respectively. Gene expression profiles in 77 breast tumors (8 DCIS and 69 IDC premenopausal patients) identified 325 genes with commonly altered expression (Figure 4A, 4B); 78 genes that were commonly up-regulated more than three-fold over their levels in normal breast ductal cells (Figure 4A, 4B, Table 3, 5), whereas 247 genes whose expression were reduced to less than 1/3 in breast cancer cells (Figure 4A, 4B, Table 4, 6). In particular, as shown in Figure 4B, expression level of 25 genes was increased and that of 49 genes was decreased from DCIS to IDC (Table 5 and 6). Among genes with elevated expression, fibronectin (*FN1*) which had already been reported as over-expressed in breast cancers (Mackay, A. et al., cDNA microarray analysis of genes associated with ERBB2 (HER2/neu) overexpression in human mammary luminal epithelial cells. Oncogene, *22:* 2680-2688, 2003.; Lalani, E. N. et al., Expression of the gene coding for a human mucin in mouse mammary tumor cells can affect their tumorigenicity. J Biol Chem, *266:* 15420-15426, 1991.; 22.Martin-Lluesma, S., et al., A. Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2. Science, *297:* 2267-2270, 2002.)was included (Table 4). On the other hand, among genes with decreased expression, *ST5* and *SCHIP1* which were known to function as tumor suppressor were also included (Table 6).

Next, we applied to identify the genes with specifically altered expression exclusively

in IDC. As a result, 24 up-regulated genes (Figure 4C, Table 7) and 41 down-regulated genes (Figure 4C, Table 8) were identified. Of the up-regulated genes, *ERBB2, CCNB1, BUB1B* which had already known to be involved in carcinogenesis of breast cancers (Latta, E. K., et al., The role of HER2/neu overexpression/amplification in the progression of ductal carcinoma in situ to invasive carcinoma of the breast. Mod Pathol, *15:* 1318-1325, 2002.; Takeno, S., et al., Prognostic value of cyclin B1 in patients with esophageal squamous cell carcinoma. Cancer, *94:* 2874-2881, 2002.; Slamon, D. J., et al., Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science, *235:* 177-182, 1987.), whereas, of down-regulated genes, *AXUD1*, a gene induced by *AXIN*, which was frequently down-regulated in lung, liver, colon and kidney cancers (Ishiguro, H., et al., Identification of AXUD1, a novel human gene induced by AXIN1 and its reduced expression in human carcinomas of the lung, liver, colon and kidney. Oncogene, *20:* 5062-5066, 2001.) was included, suggesting that *AXUD1* might be also involved in carcinogenesis of breast cancer.

Verification of selected genes by semi-quantitative RT-PCR

To confirm the reliability of the expression data obtained by cDNA microarray analysis, we performed semi-quantitative RT-PCR experiments for 3 genes (Accession No. AI261804, AA205444, AA167194) that were highly up-regulated in informative cases with well-differentiated type, and 2 genes (AA676987 and H22566) that were also highly up-regulated in informative cases with poorly-differentiated type. The RT-PCR results were highly concordant with those of the microarray analysis in the great majority of the tested cases (Figure 5, Table 9).

Identification of Genes with differentially expressed in histopathological types, and phenotypically difference in individual patients.

One goal in this study was to discover consistently up- or down-regulated genes at different phenotype in some patients. However, since breast cancer showed heterogeneous and various phenotypes, histopathological differentiation by microscopy were not clearly discerned using unsupervised classification by gene expression patterns as shown in Figure 2. To examine this observation more closely, we performed a random-permutation test and extracted the 206 genes that can distinguish between well-differentiated and poorly-

differentiated cases. These 206 discriminating genes were all significant at the level of P< 0.01 between 31 well- and 24 poorly-differentiated cancers (Figure 6, Table 10). Two-dimensional hierarchical clustering analysis using these 206 genes was also able to classify the groups with regard to the distinct components of IDC (well-differentiated, moderately-differentiated and poorly-differentiated). Group A cluster contained genes with markedly increased expression in poorly-differentiated samples (blue bar in the horizontal row); extracellular matrix structure (*COL1A2, COL3A1* and *P4HA2*), cell adhesion (*LOXL2, THBS2* and *TAGLN2*), whereas group B cluster contained the genes with increased expression primarily in well-differentiated and moderately-differentiated samples (red bar in the horizontal row); regulation of transcription (*BTF, WTAP, HTATSF1*), cell cycle regulator (*CDC5L, CCT7*). Two poorly-differentiated samples (sample # 10709 and 10781) in group B, however, showed an expression pattern that is similar to well-differentiated signature rather than poorly-differentiated types. Some well-differentiated samples demonstrated coexpression of some genes that are characteristic of the poorly-differentiated signature.

Development of Predictive Scores for Lymph Node Metastasis

In breast cancer, invasion into axillary lymph nodes is the most important prognostic factor (Shek, L. L. and Godolphin, W. Model for breast cancer survival: relative prognostic roles of axillary nodal status, TNM stage, estrogen receptor concentration, and tumor necrosis. Cancer Res, *48:* 5565-5569, 1988.). To develop an equation to achieve a scoring parameter for the prediction of axially lymph node metastasis using expression profiles of selected genes, we compared the expression profiles of 20 node-positive cases and 20 node-negative cases. Following the criteria as describe in Material and Methods, we first selected the 93 discriminating genes that showed permutation p-values of less than 0.0001. Then we obtained the top 34 genes in our candidate list that showed the best separation of node-positive from –negative cases (Table11). As shown in Figure 7A, a hierarchical clustering analysis using this 34 genes clearly classified all 40 breast cancer cases into one of two groups according to lymph-node status.

Finally, we applied to construct the predictive-scoring system that could clearly distinguish node-positive cases from node–negative cases using the expression profiles of the set of 34 genes. To further validate this scoring system, we calculated scores for 20 node-positive cases and 20 lymph node-negative cases that had not been among those used

for construction of the scoring system (see "Materials and Methods"). When 15.8 as a borderline score for 40 patients belonging to positive-metastasis group and negative were clearly separated (Figure 7B) and scores of over 15.8 as "positive", and those of 15.8 or lower as "negative". To clarify the system further, we calculated the prediction score of metastasis from primary tumors, 17 node-positive cases and 20 negative cases who had not been part of the original procedure for selecting discrimination genes. As shown in Figure 7B and 7C, among the 17 cases with lymph-node metastasis, all cases had positive scores by our definition, whereas 18 (90%) of the 20 cases without lymph-node metastasis showed negative scores. 75 (97%) cases of 77 were placed correctly according to their lymph-node status, but two node–negative cases were misplaced or placed to the borderline or positive region.

DISCUSSION

Breast cancer is a multifactor disease that develops as a result of interactions among genetic, environmental, and hormonal factors. Although distinct pathological stages of breast cancer have been described, the molecular differences among these stages are largely unknown (McGuire, W. L. Breast cancer prognostic factors: evaluation guidelines. J Natl Cancer Inst, *83:* 154-155, 1991.; Eifel, P., et al., National Institutes of Health Consensus Development Conference Statement: adjuvant therapy for breast cancer, November 1-3, 2000. J Natl Cancer Inst, *93:* 979-989, 2001.; Fisher, B., et al., Twenty-year follow-up of a randomized trial comparing total mastectomy, lumpectomy, and lumpectomy plus irradiation for the treatment of invasive breast cancer. N Engl J Med, *347:* 1233-1241, 2002.)

The development of genome-wide analysis of gene expression and laser microbeam microdissection (LMM) isolating pure cancerous cell populations of breast cancer and that origin make it possible to search molecular-target genes for cancer-specific classification, treatment and outcome prediction in a variety of tumor types, especially in breast cancer.

Since, in mammary gland tissue adipocytes account for more than 90%, and epithelial cells in the organ, from which the carcinoma originates, correspond to a very small percentage, an analysis of gene-expression profiles using whole cancer tissues and normal whole mammary gland is significantly influenced by the particular mixture of cells in the tissues examined; proportional differences of adipocytes, fibroblasts, and inflammatory cells

can mask significantly specific-expression of genes involved in breast carcinogenesis. Hence, we here used an LMM system to purify as much as possible the populations of cancerous cells and normal epithelial cells obtained from surgical specimens (Hasegawa, S., et al. Genome-wide analysis of gene expression in intestinal-type gastric cancers using a complementary DNA microarray representing 23,040 genes. Cancer Res, *62:* 7012-7017, 2002.; Kitahara, et al., and Tsunoda, T. Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia. Cancer Res, *61:* 3544-3549, 2001.; Kikuchi, T., et al. Expression profiles of non-small cell lung cancers on cDNA microarrays: identification of genes for prediction of lymph-node metastasis and sensitivity to anti-cancer drugs. Oncogene, *22:* 2192-2205, 2003.; Gjerdrum, L. M., et al., Laser-assisted microdissection of membrane-mounted paraffin sections for polymerase chain reaction analysis: identification of cell populations using immunohistochemistry and in situ hybridization. J Mol Diagn, *3:* 105-110, 2001.), (Figure 1). To evaluate the purity of microdissected cell populations, we analyzed expression of *PLIN* and *FABP4*, which are highly expressed in adipose tissue and mammary gland by our gene expression profiles in 29 normal human tissues by using a cDNA microarray (Saito-Hisaminato, A., et al., Genome-wide profiling of gene expression in 29 normal human tissues with a cDNA microarray. DNA Res, *9:* 35-45, 2002.). After the dissection procedure the proportion of contaminating adipocytes among the normal breast ductal epithelial cells was estimated to be smaller than 0.6%. In particular, when we examined expression levels of *PLIN* (Nishiu, J., et al., Isolation and chromosomal mapping of the human homolog of perilipin (PLIN), a rat adipose tissue-specific gene, by differential display method. Genomics, *48:* 254-257, 1998.), the purity of cell populations subjected to the LMM technique could therefore be approximately 100%. As shown in Figure 2, unsupervised cluster analysis represented that breast cancer whole tissues were separated from microdissected breast cancer cells by LMM, whereas normal breast ductal cells and mammary glands were clustered in same branch. Hence, to obtain accurate the breast cancer specific expression profiles of some studies, it is essential to microdissect breast cancer cells and normal breast ductal epithelial cells which originates from breast cancer. The combined use of LMM and cDNA microarray analysis provides a powerful approach to elucidate precious molecular events surrounding the development and progression of breast cancer, suggesting to lead to understand the mechanism of multistep of carcinogenesis of breast cancer cells and tumor heterogeneneity.

As shown in Figure 2A, through an unsupervised classification analysis on the basis of expression profiles, primary breast cancer can be divided into two groups and showed to associate with ER status by EIA. We found that ER+ and ER- tumors display very different gene expression phenotypes. This result suggests that these two histologically distinct lesions have different biological natures that may play an important role in carcinogenesis of breast cancer, and that ER status can be used to establish the necessity of hormone therapy in the adjuvant setting (Eifel, P., et al National Institutes of Health Consensus Development Conference Statement: adjuvant therapy for breast cancer, November 1-3, 2000. J Natl Cancer Inst, *93:* 979-989, 2001. ; Hartge, P. Genes, hormones, and pathways to breast cancer. N Engl J Med, *348:* 2352-2354, 2003.). In addition, through supervised statistical analysis, we next selected a subset of genes that were able to separate ER-positive from ER-negative to investigate hormone dependent progression and explore novel molecular-target for anti-cancer drug. We identified 97 genes whose expression is significantly different between these two groups consisting of premenopausal patients by a random permutation test (Figure 3). Among these genes, *MAP2K4* which is a centrally-placed mediator of the SAPK pathways was included. *Cyclin D1*, a gene that is strongly associated with ER expression in breast cancer in this and other studies (May, F. E. and Westley, B. R. Expression of human intestinal trefoil factor in malignant cells and its regulation by oestrogen in breast cancer cells. J Pathol, *182:* 404-413, 1997.). Estrogens are important regulators of growth and differentiation in the normal mammary gland and are also important in the development and progression of breast carcinoma (Shek, L. L. and Godolphin, W. Model for breast cancer survival: relative prognostic roles of axillary nodal status, TNM stage, estrogen receptor concentration, and tumor necrosis. Cancer Res, *48:* 5565-5569, 1988.). Estrogens regulate gene expression via ER, however the details of the estrogen effect on downstream gene targets, the role of cofactors, and cross-talk between other signaling pathways are far from fully understood. As approximately two-thirds of all breast cancers are ER+ at the time of diagnosis, the expression of the receptor has important implications for their biology and therapy. Since recently novel selective estrogen receptor modulators (SERMs) have been developing as hormonal treatment against ER-positive breast cancer patients, these genes associated with ER status might be novel potential molecular-targets for SERMs (Smith, I. E. and Dowsett, M. Aromatase inhibitors in breast cancer. N Engl J Med, *348:* 2431-2442, 2003.). These findings suggest that the comparison of expression profiles and ER-status provides useful information to

elucidate the hormonal regulation of cell proliferation and progression of ER-independent breast cancer cells.

The development and use of molecular-based therapy for breast cancer and other human malignancies will require a detailed molecular genetic analysis of patient tissues. Histological evidence suggests that several preneoplastic states exist that precede invasive breast tumors. These histological lesions include atypical ductal hyperplasia, atypical lobular hyperplasia, ductal carcinoma in situ (DCIS), and lobular carcinoma in situ (Lakhani, S. R. The transition from hyperplasia to invasive carcinoma of the breast. J Pathol, *187:* 272-278, 1999.). These lesions are thought to fall on a histological continuum between normal breast epithelium or the terminal duct lobular units from which breast cancers arise, and the final invasive breast cancer. Several models could be considered to explain the genetic abnormalities between preneoplasia and neoplasia.

We could identify variable genes that showed commonly increased or decreased expression among the pathologically discrete stages, such as comparison of between DCIS and IDC, resulting in total of 325 genes. These genes may underlie the molecular basis of the pathological grade for breast cancer, and expression levels of these genes were correlated with advanced tumor grade. We identify 78 commonly up-regulated genes (Table 3, 5) and 247 commonly down-regulated genes (Table 4, 6) in DCIS and IDC. Among up-regulated genes, *NAT1, HEC, GATA3* and *RAI3*, which have been reported to be over-expressed in breast cancer, might be already expressed in preinvasive stages (Geylan, Y. S., et al., Arylamine N-acetyltransferase activities in human breast cancer tissues. Neoplasma, *48:* 108-111, 2001.; Chen, Y., et al., HEC, a novel nuclear protein rich in leucine heptad repeats specifically involved in mitosis. Mol Cell Biol, *17:* 6049-6056, 1997.; Bertucci, F., et al., Gene expression profiling of primary breast carcinomas using arrays of candidate genes. Hum Mol Genet, *9:* 2981-2991, 2000.; Cheng, Y. and Lotan, R. Molecular cloning and characterization of a novel retinoic acid-inducible gene that encodes a putative G protein-coupled receptor. J Biol Chem, *273:* 35008-35015, 1998.).

On the other hands, *TGFBR2*, was included as down-regulated genes in this study, is known to lead to reduced malignancy (Sun, L., et al., Expression of transforming growth factor beta type II receptor leads to reduced malignancy in human breast cancer MCF-7 cells. J Biol Chem, *269:* 26449-26455, 1994.). These findings suggest that these genes might be involved in transition from DCIS to IDC.

Particularly, we identify 24 up-regulated genes (Table 5) and 41 down-regulated genes (Table 6) with elevated or decreased expression from DCIS to IDC. The list of up-regulated elements included genes encoding transcriptional factors and proteins involved in the signal transduction pathway, and in the cell cycle, and that they play an important role in invasive tumorigenesis. Over-expression of FoxM1, cyclin B1 have been reported in various tumour types. Over-expression of FoxM1 stimulates cyclin B1 expression (Leung TW, 2001). *CCNB1* is a cell cycle control protein that is required for passage through G2 and mitosis (Pines, J. and Hunter, T. Cyclins A and B1 in the human cell cycle. Ciba Found Symp, *170:* 187-196; discussion 196-204, 1992.). *TOP2A* inhibitors are widely used as chemotherapeutic agents in lung cancer treatment (Miettinen, H. E., et al., High topoisomerase IIalpha expression associates with high proliferation rate and and poor prognosis in oligodendrogliomas. Neuropathol Appl Neurobiol, *26:* 504-512, 2000.). *BUB1B* may be responsible for a chromosomal instability phenotype contributing to tumor progression in mitotic checkpoint and genetic instability (Bardelli, A., et al. Carcinogen-specific induction of genetic instability. Proc Natl Acad Sci U S A, *98:* 5770-5775, 2001.). *MMP11*, its expression was shown to have a direct negative effect on patients' survival (Boulay, A., et al. High cancer cell death in syngeneic tumors developed in host mice deficient for the stromelysin-3 matrix metalloproteinase. Cancer Res, *61:* 2189-2193, 2001.). *ECM1* has angiogenic properties and is expressed by breast tumor cells (Han, Z., et al., Extracellular matrix protein 1 (ECM1) has angiogenic properties and is expressed by breast tumor cells. Faseb J, *15:* 988-994, 2001.). Although the most of these functions are still unknown, to understand the functional analysis of these genes may indicate that these have a role to play in mediating invasive activity.

The ability of some criteria to predict disease progression and clinical outcome is, however, imperfect. Patients with more aggressive disease can benefit from adjuvant chemotherapy or hormone therapy and are currently identified according to a combination of criteria: age, the size of the tumor, axillary-node status, the histologic type and pathological grade of cancer, and hormone-receptor status. We were able to classify histologically different tumors by subset of genes, a process that provides pathologically relevant information. Most investigators have suggested that patients have a poorer prognosis.significantly higher percentage of poorly differentiated histology.

Our surprising result from this study was the remarkable similarity in the expression profiles of different histological type in each patient. Microdissection and global gene

expression analysis, we examined the question of changes in gene expression associated with invasion and prognosis, by mRNA expression profiles from breast cancer cells at well-differenciated type and poolry using supervised analysis. Through an unsupervised classification analysis on the basis of expression profiles, breast cancer can be divided into two groups and showed to associate with different pathologically lesions. We identified 25 genes whose expression is significantly different between these two groups consisting of each patient by a random permutation test (Figure 8B). Among these genes, nuclear factor of activated T-cells 5 (*NFAT5*) is restricted to promoting carcinoma cell migration, highlights the possibility of distinct genes that are induced by these transcription factors (Sebastien J. et al., The role of NFAT transcription factors in integrin-mediated carcinoma invasion. Nature cell biology, *4:* 540-544, 2002.). Thrombospondin 2 (*THSB2*) is extracellular matrix proteins that appear to play a role in cell adhesion and cell migration. One important advantage of our LMM-based approach is the ability to select cancer cells of different phenotypes from the one specimen. Systematic analysis of gene-expression patterns provides a window on the biology and pathogenesis of invasion.

Furthermore, lymph-node metastasis is a critical step in tumor progression and one of the major cause of prognosis in breast cancer patients (Shek, L. L. and Godolphin, W. Model for breast cancer survival: relative prognostic roles of axillary nodal status, TNM stage, estrogen receptor concentration, and tumor necrosis. Cancer Res, *48:* 5565-5569, 1988.), but only a minority of patients exhibits clinically detectable metastases at diagnosis. Lymph-node status at diagnosis is the most important measure for future recurrence and overall survival, it is a surrogate that is imperfect at best. About a third of patients with no detectable lymph-node involvement, for example, will develop recurrent disease within 10 years (Saphner, T., et al., Annual hazard rates of recurrence for breast cancer after primary therapy. J Clin Oncol, *14:* 2738-2746, 1996.). Sentinel lymph node biopsy was shown to be an accurate procedure in the study of axillary lymph nodes; it allowed a marked decrease in surgery-related morbidity of breast cancer and axillary dissection could be avoided. Other parameters as nuclear grading, patient age, tumor size, are not able to predict the axillary lymph node status, and it is not perfectly to diagnose lymph node status by sentinel lymph node biopsy. Therefore, our identification of a subset of genes that were differently expressed between node-positive and node-negative tumors can contribute to improve clinical diagnosis and understand the precise biophysical events. Cluster analysis (Figure.9A, B) suggested to separate cases with lymph-

node metastasis from those without metastasis. This subset of genes that contributed to separation of our two patient groups according to the status of lymph-node metastasis might serve as molecular markers for metastasis (Ramaswamy, S., et al., A molecular signature of metastasis in primary solid tumors. Nat Genet, *33:* 49-54, 2003.). For example, among these 34 genes, FUS which is known as TLS for translocated in liposarcoma, is decreased in node-negative cancers is translocated with the gene encoding the transcription factor ERG-1 in human myeloid leukaemias. One of the functions of wild-type FUS is important in genome maintenance of genomic stability (Hicks, G. G., et al., Fus deficiency in mice results in defective B-lymphocyte development and activation, high levels of chromosomal instability and perinatal death. Nat Genet, *24:* 175-179, 2000.). Expression levels were increased for some of those genes in metastasis-positive group as compared to the negative group; *EEF1D*, The higher expression of EF-1 delta in the tumours suggested that malignant transformation in vivo requires an increase in translation factor mRNA and protein synthesis for entry into and transition through the cell cycle. *CFL1*, Rho protein signal transduction, Rho family GTPases regulate the cytoskeleton and cell migration and are frequently overexpressed in tumours (Yoshizaki, H., et al., Activity of Rho-family GTPases during cell division as visualized with FRET-based probes. J Cell Biol, *162:* 223-232, 2003.; Arthur, W. T., et al., Regulation of Rho family GTPases by cell-cell and cell-matrix adhesion. Biol Res, *35:* 239-246, 2002.). *BRAF*, the B-Raf kinase was shown to be capable of phosphorylating and activating MEK as a result of growth factor stimulation. Although the function of them is still unknown, to understand the functional analysis of these gene products may clarify their roles in metastasis in breast cancer.

The causes and clinical course of recurrence, and it is not possible to predict outcome reliably on the basis of available clinical, pathological, and genetic markers. Although we believe that this predicting score system using the expression profiles of these 34 genes might be useful for improvement of prognosis, verification using a larger number of cases is essential for introduction of clinical stages. That indicates a potential for providing precious information about the biological nature of cancer cells that misunderstood by conventional histological diagnosis.

Cancer therapies directed at specific molecular alterations that occur in cancer cells have been validated through clinical development and regulatory approval of anti-cancer drugs such as trastuzumab (Herceptin) for the treatment of advanced breast cancer (Coussens,

L., et al. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. Science, *230:* 1132-1139, 1985.). This drug is clinically effective and better tolerated than traditional anti-cancer agents because they target only transformed cells. Hence, this drug not only improves survival and quality of life for cancer patients, but also validates the concept of molecularly targeted cancer therapy. Furthermore, targeted drugs can enhance the efficacy of standard chemotherapy when used in combination with it (Gianni, L. and Grasselli, G. Targeting the epidermal growth factor receptor a new strategy in cancer treatment. Suppl Tumori, *1:* S60-61, 2002.; Klejman, A., et al., Phosphatidylinositol-3 kinase inhibitors enhance the anti-leukemia effect of STI571. Oncogene, *21:* 5868-5876, 2002.). Therefore, future cancer treatments will probably involve combining conventional drugs with target-specific agents aimed at different characteristics of tumor cells such as angiogenesis and invasiveness. Furthermore, this study would be made the novel tumor markers, substances that may be present in abnormal amounts in the blood, or nipple aspirates of a woman who has breast cancer, is reliable enough to be used routinely to detect early breast cancer.

Currently, no effective treatment is available for patients in advanced breast cancer. Thus, new therapeutic approaches and tailor-made treatment are urgently required. Our cancer-specific expression profile include up- or down-regulated genes in breast cancers should provide useful information for identifying molecular targets for the treatment of patents.

Table 1 List of genes with altered expression between well and poorly differentiated type in histological phenotype

	ACCESSION	Symbol	TITLE	p-value
1	AF053712	TNFSF11	tumor necrosis factor (ligand) superfamily, member 11	1.2E-06
2	BF973104	LOC201725	hypothetical protein LOC201725	3.2E-05
3	AV752313	KPNA6	karyopherin alpha 6 (importin alpha 7)	1.1E-04
4	AK026898	FOXP1	forkhead box P1	7.4E-04
5	AA148107	ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	7.9E-04
6	AK001067	NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	8.2E-04
7	AB007919	KIAA0450	KIAA0450 gene product	1.8E-03
8	BG026429	SFRS2	splicing factor, arginin /s rin -rich 2	2.0E-03

9	M87770	FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)	2.1E-03
10	L02785	SLC26A3	solute carrier family 26, member 3	2.7E-03
11	BF037402		Homo sapiens, clone MGC:17296 IMAGE:3460701, mRNA, complete cds	2.8E-03
12	L12350	THBS2	thrombospondin 2	2.8E-03
13	N36875		Homo sapiens, clone IMAGE:4994678, mRNA	3.8E-03
14	AL135342		ESTs, Weakly similar to neuronal thread protein [Homo sapiens] [H.sapiens]	4.3E-03
15	AL049426	SDC3	syndecan 3 (N-syndecan)	4.5E-03
16	AW961424	KIAA1870	KIAA1870 protein	5.2E-03
17	AA523117	DC-TM4F2	tetraspanin similar to TM4SF9	5.5E-03
18	Z11531	EEF1G	eukaryotic translation elongation factor 1 gamma	6.1E-03
19	AI423028	SMARCD3	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3	6.8E-03
20	AB002391	MN7	D15F37 (pseudogene)	7.1E-03
21	D32050	AARS	alanyl-tRNA synthetase	7.2E-03
22	BE876949	RAB7	RAB7, member RAS oncogene family	7.9E-03
23	AW291083		ESTs	8.0E-03
24	AI568910		ESTs	8.2E-03
25	AK023480	SRP72	signal recognition particle 72kDa	8.7E-03

Table2 List of genes with altered expression between ER-positive and ER-negative tumors

	ACCESSION	Symbol	TITLE	p-value
26	AW949747	GATA3	GATA binding protein 3	3.2E-20
27	BE868254	ESTs	ESTs	2.2E-14
28	AF037335	CA12	carbonic anhydrase XII	1.6E-13
29	BF724977	ASB13	ankyrin repeat and SOCS box-containing 13	8.5E-13
30	NM_004636	SEMA3B	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	9.7E-13
31	NM_000125	ESR1	estrogen receptor 1	1.2E-12
32	M73554	CCND1	cyclin D1 (PRAD1: parathyroid adenomatosis 1)	3.9E-12
33	NM_005544	IRS1	insulin receptor substrate 1	4.4E-12
34	M14745	BCL2	B-cell CLL/lymphoma 2	5.1E-12
35	BE826171	BCMP11	breast cancer membrane protein 11	2.8E-11
36	AI087270	SIAH2	seven in absentia homolog 2 (Drosophila)	2.8E-11
37	L07033	HMGCL	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyas (hydroxymethylglutaricaciduria)	2.8E-11
38	AB014523	ULK2	unc-51-like kinase 2 (C. legans)	4.0E-11
39	AL137588	DKFZp434K1210	hypothetical protein DKFZp434K1210	5.2E-11

40	AL137566	EST	Homo sapiens mRNA; cDNA DKFZp586G0321 (from clone DKFZp586G0321)	5.4E-11
41	AF038421	GFRA1	GDNF family r ceptor alpha 1	8.4E-11
42	AI194045	FE65L2	FE65-like protein 2	9.2E-11
43	BG163478	ESTs	ESTs, Weakly similar to BAI1_HUMAN Brain-specific angiogenesis inhibitor 1 precursor [H.sapiens]	1.1E-10
44	M31627	XBP1	X-box binding protein 1	1.1E-10
45	AA156269	EST	Homo sapiens, clone IMAGE:4794107, mRNA	1.3E-10
46	NM_006763	BTG2	BTG family, member 2	1.9E-10
47	AW504052	SEC15L	SEC15 (S. cerevisiae)-like	2.1E-10
48	NM_005400	PRKCE	protein kinase C, epsilon	2.3E-10
49	AI628151	XBP1	X-box binding protein 1	2.7E-10
50	AF043045	FLNB	filamin B, beta (actin binding protein 278)	3.5E-10
51	U31383	GNG10	guanine nucleotide binding protein (G protein), gamma 10	4.6E-10
52	L10333	RTN1	reticulon 1	5.6E-10
53	AK025099	SIGIRR	single Ig IL-1R-related molecule	6.2E-10
54	AL039253	LIV-1	LIV-1 protein, estrogen regulated	7.4E-10
55	AW949662	KIAA0239	KIAA0239 protein	8.0E-10
56	D13629	KTN1	kinectin 1 (kinesin receptor)	1.5E-09
57	NM_000165	GJA1	gap junction protein, alpha 1, 43kDa (connexin 43)	1.5E-09
58	AA533079	C1orf21	chromosome 1 open reading frame 21	1.8E-09
59	AF251056	CAPS2	calcyphosphine 2	1.9E-09
60	AF061016	UGDH	UDP-glucose dehydrogenase	2.0E-09
61	U92544	MAGED2	melanoma antigen, family D, 2	2.1E-09
62	BE617536	RPL13A	ribosomal protein L13a	2.4E-09
63	AK024102	MYST1	MYST histone acetyltransferase 1	2.5E-09
64	BF212902	EST	Homo sapiens mRNA; cDNA DKFZp564F053 (from clone DKFZp564F053)	2.8E-09
65	AK025480	FLJ21827	hypothetical protein FLJ21827	3.0E-09
66	AI376713	ESTs	ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]	3.6E-09
67	AI028483	ESTs	ESTs	3.8E-09
68	AK022249	EST.	Homo sapiens cDNA FLJ12187 fis, clone MAMMA1000831.	4.2E-09
69	AI568527	EST	Homo sapiens cDNA FLJ34849 fis, clone NT2NE2011687.	5.0E-09
70	AL133074	TP53INP1	tumor protein p53 inducible nuclear protein 1	5.3E-09
71	AF022116	PRKAB1	protein kinase, AMP-activated, beta 1 non-catalytic subunit	6.1E-09
72	AF007170	C1orf34	chromosome 1 open reading frame 34	9.7E-09
73	AF042081	SH3BGRL	SH3 domain binding glutamic acid-rich protein like	1.2E-08
74	AK027813	MGC10744	hypothetical protein MGC10744	1.4E-08
75	M57609	GLI3	GLI-Kruppel family member GLI3 (Greig cephalopolysyndactyly syndrome)	1.7E-08
76	AL359600	EST	Homo sapiens mRNA; cDNA DKFZp547C136 (from clone DKFZp547C136)	1.9E-08

77	BQ006049	TIMP1	tissue inhibitor of metalloproteinas 1 (erythroid potentiating activity, collag nas inhibitor)	2.1E-08
78	AF111849	HELO1	homolog of y ast long chain polyunsaturated fatty acid elongation enzyme 2	2.2E-08
79	AL157499	RAB5EP	rabaptin-5	2.2E-08
80	AK023199	EST	Homo sapiens cDNA FLJ13137 fis, clone NT2RP3003150.	2.5E-08
81	J05176	SERPINA3	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	3.2E-08
82	AA028101	KIAA0303	KIAA0303 protein	3.3E-08
83	AI300588	MAP2K4	mitogen-activated protein kinase kinase 4	4.1E-08
84	AA682861	ESTs	ESTs, Moderately similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]	4.6E-08
85	M26393	ACADS	acyl-Coenzyme A dehydrogenase, C-2 to C-3 short chain	5.4E-08
86	NM_001609	ACADSB	acyl-Coenzyme A dehydrogenase, short/branched chain	5.5E-08
87	U91543	CHD3	chromodomain helicase DNA binding protein 3	5.7E-08
88	AK023813	FLJ10081	hypothetical protein FLJ10081	6.0E-08
89	BF111711	FLJ20727	hypothetical protein FLJ20727	7.0E-08
90	AL049987	EST	Homo sapiens mRNA; cDNA DKFZp564F112 (from clone DKFZp564F112)	7.2E-08
91	AW081894	EST	EST	8.2E-08
92	AK000350	FLJ20343	hypothetical protein FLJ20343	1.1E-07
93	AA418493	DPP7	dipeptidylpeptidase 7	1.1E-07
94	BE674061	PIN4	protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting, 4 (parvulin)	1.2E-07
95	AB011155	DLG5	discs, large (Drosophila) homolog 5	1.2E-07
96	L15203	TFF3	trefoil factor 3 (intestinal)	1.4E-07
97	NM_001552	IGFBP4	insulin-like growth factor binding protein 4	1.4E-07
98	M57230	IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	1.5E-07
99	N92706	EST	Homo sapiens cDNA FLJ38461 fis, clone FEBRA2020977.	1.5E-07
100	M30704	AREG	amphiregulin (schwannoma-derived growth factor)	1.8E-07
101	AB004066	BHLHB2	basic helix-loop-helix domain containing, class B, 2	2.2E-07
102	M15518	PLAT	plasminogen activator, tissue	2.3E-07
103	BM697477	ShrmL	Shroom-related protein	2.4E-07
104	R45979	CELSR1	cadherin, EGF LAG seven-pass G-type receptor 1 (flamingo homolog, Drosophila)	3.0E-07
105	AL049365	EST	Homo sapiens mRNA; cDNA DKFZp586A0618 (from clone DKFZp586A0618)	6.5E-07
106	NM_003225	TFF1	trefoil factor 1 (breast cancer, estrogen-inducible sequenc expr ssed in)	7.1E-07
107	AI733356	EST	Homo sapiens cDNA FLJ31746 fis, clon NT2RI2007334.	7.8E-07
108	AF078853	KIAA1243	KIAA1243 protein	8.2E-07

109	N30179	PLAB	prostate differentiation factor	1.0E-06
110	BG026429	SFRS2	splicing factor, arginine/serine-rich 2	2.4E-06
111	AU149272	ESTs	ESTs	2.5E-06
112	J03827	NSEP1	nuclease sensitive element binding protein 1	3.0E-06
113	AJ276469	C20orf35	chromosome 20 open reading frame 35	3.4E-06
114	AW295100	LOC201562	hypothetical protein LOC201562	3.9E-06
115	J03817	GSTM1	glutathione S-transferase M1	4.8E-06
116	AF288571	LEF1	lymphoid enhancer-binding factor 1	5.1E-06
117	AF069301	PECI	peroxisomal D3,D2-enoyl-CoA isomerase	5.3E-06
118	AA621665	EST	EST	6.7E-06
119	AI739486	ESTs	ESTs	8.0E-06
120	X81438	AMPH	amphiphysin (Stiff-Man syndrome with breast cancer 128kDa autoantigen)	8.7E-06
121	U89606	PDXK	pyridoxal (pyridoxine, vitamin B6) kinase	8.8E-06
122	NM_017555	EGLN2	egl nine homolog 2 (C. elegans)	9.2E-06

Table 3 Genes commonly up-regulated in DCIS and IDC

	ACCESSION	Symbol	TITLE
123	D90041	NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)
124	M13755	G1P2	interferon, alpha-inducible protein (clone IFI-15K)
125	D88308	SLC27A2	solute carrier family 27 (fatty acid transporter), member 2
126	AW235061	SLC1A1	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1
127	K02215	AGT	angiotensinogen (serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 8)
128	AB032261	SCD	stearoyl-CoA desaturase (delta-9-desaturase)
129	NM_000909	NPY1R	neuropeptide Y receptor Y1
130	AF017790	HEC	highly expressed in cancer, rich in leucine heptad repeats
131	NM_007019	UBE2C	ubiquitin-conjugating enzyme E2C
132	AF065388	TSPAN-1	tetraspan 1
133	N70334	DUSP10	dual specificity phosphatase 10
134	AA621719	SMC4L1	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)
135	AA676987		ESTs
136	AK001402	C10orf3	chromosome 10 open reading frame 3
137	AW949747	GATA3	GATA binding protein 3
138	AK001472	ANLN	anillin, actin binding protein (scraps homolog, Drosophila)
139	AA789233	COL1A1	collagen, type I, alpha 1
140	AF070632		Homo sapiens clone 24405 mRNA sequence
141	H04544	NPY1R	neuropeptide Y receptor Y1
142	AI015982	CDCA1	cell division cycle associated 1
143	NM_003979	RAI3	retinoic acid induced 3
144	BF516445	CLIC6	chloride intracellular channel 6
145	AI361654		
146	AI077540		Homo sapiens cDNA FLJ38379 fis, clon FEBRA2002986.
147	AI261804		Homo sapi ns MSTP020 (MST020) mRNA, complete cds
148	AK026559	TPM3	tropomyosin 3
149	J03473	ADPRT	ADP-ribosyltransferase (NAD+; poly (ADP-ribose) polymeras)

150	NM_000187	HGD	homogentisate 1,2-dioxygenas (homogentisat oxidase)
151	L43964	PSEN2	pr senilin 2 (Alzheimer diseas 4)
152	J05581	MUC1	mucin 1, transmembran
153	AA602499	GLCCI1	glucocorticoid induced transcript 1
154	U37707	MPP3	membrane protein, palmitoylated 3 (MAGUK p55 subfamily member 3)
155	AB030905	CBX3	chromobox homolog 3 (HP1 gamma homolog, Drosophila)
156	AL138409		Homo sapiens mRNA; cDNA DKFZp313L231 (from clone DKFZp313L231)
157	AV756928	SEC61G	Sec61 gamma
158	AI205684	HSPA2	heat shock 70kDa protein 2
159	BE739464	ARL6IP	ADP-ribosylation factor-like 6 interacting protein
160	AI081356	LOC253782	hypothetical protein LOC253782
161	AA167194	LOC253782	hypothetical protein LOC253782
162	M90516	GFPT1	glutamine-fructose-6-phosphate transaminase 1
163	AL133074	TP53INP1	tumor protein p53 inducible nuclear protein 1
164	AL137257		Homo sapiens, clone IMAGE:5296692, mRNA
165	AK025240	LOC223082	LOC223082
166	AJ007042	WHSC1	Wolf-Hirschhorn syndrome candidate 1
167	U42068	GRP58	glucose regulated protein, 58kDa
168	AJ132592	ZNF281	zinc finger protein 281
169	W93638		ESTs
170	AW977394	C9orf12	chromosome 9 open reading frame 12
171	AI347925	HSPB1	heat shock 27kDa protein 1
172	AK026587	NET-6	transmembrane 4 superfamily member tetraspan NET-6
173	AI264621	LASS2	LAG1 longevity assurance homolog 2 (S. cerevisiae)
174	AA767828	FLJ10980	hypothetical protein FLJ10980
175	AU142881	FLJ10702	hypothetical protein FLJ10702

Table 4 Genes commonly down-regulated in DCIS and IDC

	ACCESSION	Symbol	TITLE
176	X52186	ITGB4	integrin, beta 4
177	NM_006297	XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1
178	X73460	RPL3	ribosomal protein L3
179	NM_001436	FBL	fibrillarin
180	X59373	HOXD10	homeo box D10
181	J04208	IMPDH2	IMP (inosine monophosphate) dehydrogenase 2
182	L24203	TRIM29	tripartite motif-containing 29
183	L10340	EEF1A2	eukaryotic translation elongation factor 1 alpha 2
184	J04621	SDC2	syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan)
185	L08424	ASCL1	achaete-scute complex-like 1 (Drosophila)
186	AI376713	EST	ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]
187	AK026966	EST	Homo sapiens cDNA: FLJ23313 fis, clon HEP11919.
188	NM_001050	SSTR2	somatostatin receptor 2
189	AA632025	EST	ESTs

190	N22918	FLJ32332	hypothetical protein FLJ32332
191	AF272043	ITM2C	integral m mbrane protein 2C
192	M58459	RPS4Y	ribosomal protein S4, Y-linked
193	AI133697	EST	Homo sapiens, clone MGC:16362 IMAGE:3927795, mRNA, complete cds
194	AA780301	CTSF	cathepsin F
195	M92843	ZFP36	zinc finger protein 36, C3H type, homolog (mouse)
196	AA570186	EST	Human full-length cDNA 5-PRIME end of clone CS0DK007YB08 of HeLa cells of Homo sapiens (human)
197	R56906	EST	EST
198	AF208860	TNFRSF21	tumor necrosis factor receptor superfamily, member 21
199	AK025216	TAZ	transcriptional co-activator with PDZ-binding motif (TAZ)
200	AA758394	PTPN1	protein tyrosine phosphatase, non-receptor type 1
201	AA628530	ISYNA1	myo-inositol 1-phosphate synthase A1
202	AF161416	IRS2	insulin receptor substrate 2
203	AL045916	EST	ESTs
204	AW340972	EST	Homo sapiens cDNA: FLJ22864 fis, clone KAT02164.
205	AI189414	RNPC2	RNA-binding region (RNP1, RRM) containing 2
206	AV705636	EIF3S6IP	eukaryotic translation initiation factor 3, subunit 6 interacting protein
207	U28977	CASP4	caspase 4, apoptosis-related cysteine protease
208	AV708528	MSCP	mitochondrial solute carrier protein
209	AA022956	FLJ12750	hypothetical protein FLJ12750
210	AI928443	EST	Homo sapiens cDNA FLJ38855 fis, clone MESAN2010681.
211	U14966	RPL5	ribosomal protein L5
212	AI857997	TPBG	trophoblast glycoprotein
213	BF697545	MGP	matrix Gla protein
214	AW575754	FLJ35564	hypothetical protein FLJ35564
215	AI352534	CAV1	caveolin 1, caveolae protein, 22kDa
216	NM_001985	ETFB	electron-transfer-flavoprotein, beta polypeptide
217	AI743134	SERPINE2	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2
218	AW444709	CD47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)
219	BF688910	COPEB	core promoter element binding protein
220	AI818579	EST	Homo sapiens, clone IMAGE:3625286, mRNA, partial cds
221	S95936	TF	transferrin
222	AF074393	RPS6KA5	ribosomal protein S6 kinase, 90kDa, polypeptide 5
223	NM_000591	CD14	CD14 antigen
224	AK027181	IBA2	ionized calcium binding adapter molecule 2
225	X73079	PIGR	polymeric immunoglobulin receptor
226	NM_001343	DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)
227	M31452	C4BPA	compl ment component 4 binding protein, alpha
228	X07696	KRT15	keratin 15
229	AF016004	GPM6B	glycoprot in M6B
230	NM_004078	CSRP1	cysteine and glycine-rich protein 1

231	L36645	EPHA4	EphA4
232	D78011	DPYS	dihydropyrimidinas
233	W60630	JAM3	junctional adh sion molecule 3
234	AW956111	D4S234E	DNA segment on chromosome 4 (unique) 234 expressed sequence
235	AF035752	CAV2	caveolin 2
236	D37766	LAMB3	laminin, beta 3
237	U66406	EFNB3	ephrin-B3
238	X52001	EDN3	endothelin 3
239	NM_000856	GUCY1A3	guanylate cyclase 1, soluble, alpha 3
240	U60115	FHL1	four and a half LIM domains 1
241	D14520	KLF5	Kruppel-like factor 5 (intestinal)
242	M99487	FOLH1	folate hydrolase (prostate-specific membrane antigen) 1
243	U09873	FSCN1	fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus)
244	AF017418	MEIS2	Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse)
245	AF038540	RTN2	reticulon 2
246	AF049884	ARGBP2	Arg/Abl-interacting protein ArgBP2
247	NM_001122	ADFP	adipose differentiation-related protein
248	Y09926	MASP2	mannan-binding lectin serine protease 2
249	M58297	ZNF42	zinc finger protein 42 (myeloid-specific retinoic acid-responsive)
250	AF035811	PNUTL2	peanut-like 2 (Drosophila)
251	L22214	ADORA1	adenosine A1 receptor
252	AF177775	CES1	carboxylesterase 1 (monocyte/macrophage serine esterase 1)
253	U07643	LTF	lactotransferrin
254	S76474	NTRK2	neurotrophic tyrosine kinase, receptor, type 2
255	BE299605	MRAS	muscle RAS oncogene homolog
256	NM_006225	PLCD1	phospholipase C, delta 1
257	NM_005036	PPARA	peroxisome proliferative activated receptor, alpha
258	M22324	ANPEP	alanyl (membrane) aminopeptidase (aminopeptidase N, aminopeptidase M, microsomal aminopeptidase, CD13, p150)
259	BE877416	TGFBR2	transforming growth factor, beta receptor II (70/80kDa)
260	BE561244	RPL18A	ribosomal protein L18a
261	AL048962	EST	Homo sapiens, clone IMAGE:4243767, mRNA
262	L08895	MEF2C	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)
263	U48707	PPP1R1A	protein phosphatase 1, regulatory (inhibitor) subunit 1A
264	X56134	RPLP2	ribosomal protein, large P2
265	D84239	FCGBP	Fc fragment of IgG binding protein
266	AK026181	PHLDA1	pleckstrin homology-like domain, family A, member 1
267	K01144	CD74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)
268	U25138	KCNMB1	potassium larg conductance calcium-activated channel, subfamily M, beta memb r 1
269	X85337	MYLK	myosin, light polypeptide kinase

270	D83597	LY64	lymphocyte antig n 64 homolog, radioprotective 105kDa (mouse)
271	NM_004024	ATF3	activating transcription factor 3
272	BF126636	SAA1	serum amyloid A1
273	D13789	MGAT3	mannosyl (beta-1,4-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase
274	L41142	STAT5A	signal transducer and activator of transcription 5A
275	AB040969	KIAA1536	KIAA1536 protein
276	NM_002153	HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2
277	AV646610	ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein
278	X03663	CSF1R	colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog
279	U47025	PYGB	phosphorylase, glycogen; brain
280	M81349	SAA4	serum amyloid A4, constitutive
281	AI264201	EGR2	early growth response 2 (Krox-20 homolog, Drosophila)
282	U18018	ETV4	ets variant gene 4 (E1A enhancer binding protein, E1AF)
283	NM_004350	RUNX3	runt-related transcription factor 3
284	BF337516	CRYAB	crystallin, alpha B
285	AF027208	PROML1	prominin-like 1 (mouse)
286	D17408	CNN1	calponin 1, basic, smooth muscle
287	NM_004010	DMD	dystrophin (muscular dystrophy, Duchenne and Becker types)
288	BF183952	CSTA	cystatin A (stefin A)
289	M16445	CD2	CD2 antigen (p50), sheep red blood cell receptor
290	AF055015	EYA2	eyes absent homolog 2 (Drosophila)
291	AI745624	ELL2	ELL-related RNA polymerase II, elongation factor
292	AK025329	DKFZP566H073	DKFZP566H073 protein
293	BE745465	KLK5	kallikrein 5
294	AK024578	DKFZP761F241	hypothetical protein DKFZp761F241
295	AI870306	IRX1	iroquois homeobox protein 1
296	H37853	C9orf19	chromosome 9 open reading frame 19
297	BF000047	EST	Homo sapiens full length insert cDNA clone ZA79C08
298	AF126780	RetSDR2	retinal short-chain dehydrogenase/reductase 2
299	AI700341	EST	ESTs, Weakly similar to hypothetical protein FLJ20489 [Homo sapiens] [H.sapiens]
300	M87770	FGFR2	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor, craniofacial dysostosis 1, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss syndrome)
301	AA452368	FLJ30046	hypothetical protein FLJ30046
302	NM_021200	PLEKHB1	pleckstrin homology domain containing, family B (evectins) member 1
303	AK026343	hIAN2	human immune associated nucl otide 2
304	AF251040	C5orf6	chromosom 5 open reading frame 6
305	M87507	CASP1	caspase 1, apoptosis-related cyst in proteas (int rleukin 1, beta, conv rtase)

306	M97675	ROR1	receptor tyrosine kinas -like orphan receptor 1
307	NM_020549	CHAT	cholin acetyltransferase
308	X00457	HLA-DPA1	major histocompatibility complex, class II, DP alpha 1
309	W72411	TP73L	tumor protein p73-like
310	AI769569	EST	ESTs
311	K02765	C3	complement component 3
312	AW971490	FLJ14906	hypothetical protein FLJ14906
313	AF077044	RPAC2	likely ortholog of mouse RNA polymerase 1-3 (16 kDa subunit)
314	H70803	KIAA0790	KIAA0790 protein
315	AL050367	LOC221061	hypothetical protein LOC221061
316	AK001643	FLJ10781	hypothetical protein FLJ10781
317	AW182273	EST	Homo sapiens cDNA FLJ31517 fis, clone NT2RI2000007.
318	W67951	EST	Human S6 A-5 mRNA expressed in chromosome 6-suppressed melanoma cells.
319	AL117605	EST	Homo sapiens mRNA; cDNA DKFZp564N1063 (from clone DKFZp564N1063)
320	AI376418	EST	Homo sapiens cDNA FLJ35169 fis, clone PLACE6012908.
321	AA683373	EST	EST
322	AK022877	EST	Homo sapiens cDNA FLJ12815 fis, clone NT2RP2002546.
323	NM_002258	KLRB1	killer cell lectin-like receptor subfamily B, member 1
324	M69225	BPAG1	bullous pemphigoid antigen 1, 230/240kDa
325	AW299572	EHZF	early hematopoietic zinc finger
326	BE044467	ARL7	ADP-ribosylation factor-like 7
327	AA938297	FLJ20716	hypothetical protein FLJ20716
328	AA706316	ZD52F10	hypothetical gene ZD52F10
329	AI827230	APCDD1	adenomatosis polyposis coli down-regulated 1
330	AK000251	FLJ20244	hypothetical protein FLJ20244
331	N62352	KIAA1573	KIAA1573 protein
332	H53164	ICSBP1	interferon consensus sequence binding protein 1
333	BE394824	WFDC2	WAP four-disulfide core domain 2
334	AL117462	ZFP385	likely ortholog of mouse zinc finger protein 385
335	NM_003186	TAGLN	transgelin
336	U58514	CHI3L2	chitinase 3-like 2
337	AB026125	ART-4	ART-4 protein
338	AL080059	KIAA1750	KIAA1750 protein
339	AA747005	SDCCAG43	serologically defined colon cancer antigen 43
340	NM_005928	MFGE8	milk fat globule-EGF factor 8 protein
341	D62470	NRXN3	neurexin 3
342	N29574	RAGD	Rag D protein
343	K02276	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
344	D78611	MEST	mesoderm specific transcript homolog (mouse)
345	NM_022003	FXYD6	FXYD domain containing ion transport regulator 6
346	BF508973	RPL13	ribosomal prot in L13
347	NM_001615	ACTG2	actin, gamma 2, smooth muscle, nteric
348	R41532	EST	ESTs, Weakly similar to POL2_MOUSE Retrovirus-r lated POL polyprot in [Contains: Reverse transcriptas ; Endonucl as] [M.musculus]

349	AA142875	EST	ESTs
350	U03688	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
351	W94363	EST	Homo sapiens full length insert cDNA clone ZE12G01
352	W44613	HSJ001348	cDNA for differentially expressed CO16 gene
353	AL118812	EST	Homo sapiens mRNA; cDNA DKFZp761G1111 (from clone DKFZp761G1111)
354	D56064	MAP2	microtubule-associated protein 2
355	BF966838	KIAA2028	similar to PH (pleckstrin homology) domain
356	AI338625	FJX1	four jointed box 1 (Drosophila)
357	AI263022	EST	ESTs
358	AL050107	TAZ	transcriptional co-activator with PDZ-binding motif (TAZ)
359	AI056364	FLJ14855	hypothetical protein FLJ14855
360	AI351898	DRCTNNB1A	down-regulated by Ctnnb1, a
361	AV700003	ARL6IP2	ADP-ribosylation-like factor 6 interacting protein 2
362	NM_000700	ANXA1	annexin A1
363	M81141	HLA-DQB1	major histocompatibility complex, class II, DQ beta 1
364	AI598227	FLJ23091	hypothetical protein FLJ23091
365	BG034740	ROPN1	ropporin, rhophilin associated protein 1
366	AB011175	TBC1D4	TBC1 domain family, member 4
367	AK024449	PP2135	PP2135 protein
368	AW978770	DKFZP566A1524	hypothetical protein DKFZp566A1524
369	AI821113	EST	Homo sapiens cDNA FLJ36327 fis, clone THYMU2005748.
370	AI057450	SLC13A2	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2
371	X86693	SPARCL1	SPARC-like 1 (mast9, hevin)
372	AI224952	FLJ40906	hypothetical protein FLJ40906
373	D13639	CCND2	cyclin D2

Table 5 Genes with elevated expression from DCIS to IDC

	ACCESSION	Symbol	TITLE
374	U74612	FOXM1	forkhead box M1
375	U63743	KIF2C	kinesin family member 2C
376	D88532	PIK3R3	phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (p55, gamma)
377	NM_005532	IFI27	interferon, alpha-inducible protein 27
378	D14657	KIAA0101	KIAA0101 gene product
379	AF030186	GPC4	glypican 4
380	Z11566	STMN1	stathmin 1/oncoprotein 18
381	U90914	CPD	carboxypeptidase D
382	NM_002534	OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa
383	S67310	BF	B-factor, properdin
384	AA192445	TMEPAI	transmembrane, prostate androgen induced RNA
385	AB003103	PSMD12	proteasome (prosome, macropain) 26S subunit, non-ATPas , 12
386	BE878057	DKFZP564K0822	hypothetical protein DKFZp564K0822
387	AB003698	CDC7L1	CDC7 c ll division cycle 7-like 1 (S. cerevisiae)

388	M91670	E2-EPF	ubiquitin carri r prot in
389	AK023414	FLJ13352	hypothetical protein FLJ13352
390	L09235	ATP6V1A1	ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A, isoform 1
391	AF007152	ABHD3	abhydrolase domain containing 3
392	U33632	KCNK1	potassium channel, subfamily K, member 1
393	AA621719	SMC4L1	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)
394	AF176228	DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
395	H22566	DACH	dachshund homolog (Drosophila)
396	AI185804	FN1	fibronectin 1
397	AI189477	IDH2	isocitrate dehydrogenase 2 (NADP+), mitochondrial
398	AA205444	AP1S2	adaptor-related protein complex 1, sigma 2 subunit

Table 6 Genes with decreasd expression from DCIS to IDC

	ACCESSION	Symbol	TITLE
399	AF070609	SLC1A3	solute carrier family 1 (glial high affinity glutamate transporter), member 3
400	U85267	DSCR1	Down syndrome critical region gene 1
401	NM_005397	PODXL	podocalyxin-like
402	D13811	AMT	aminomethyltransferase (glycine cleavage system protein T)
403	X53586	ITGA6	integrin, alpha 6
404	L13288	VIPR1	vasoactive intestinal peptide receptor 1
405	M12125	TPM2	tropomyosin 2 (beta)
406	M65066	PRKAR1B	protein kinase, cAMP-dependent, regulatory, type I, beta
407	AJ001183	SOX10	SRY (sex determining region Y)-box 10
408	AW241712	MXI1	MAX interacting protein 1
409	AL160111	KIAA1649	KIAA1649 protein
410	X93920	DUSP6	dual specificity phosphatase 6
411	AF132734	SEC8	secretory protein SEC8
412	AI133467		ESTs
413	D88153	HYA22	HYA22 protein
414	AF014404	PTE1	peroxisomal acyl-CoA thioesterase
415	BE907755	C16orf5	chromosome 16 open reading frame 5
416	AA135341	GCN5L2	GCN5 general control of amino-acid synthesis 5-like 2 (yeast)
417	AL110126		Homo sapiens mRNA; cDNA DKFZp564H1916 (from clone DKFZp564H1916)
418	BE254330		Homo sapiens mRNA; cDNA DKFZp564D016 (from clone DKFZp564D016)
419	BE264353	RBP1	retinol binding protein 1, cellular
420	W75991		Homo sapiens, clone IMAGE:4249217, mRNA
421	AF091434	PDGFC	platelet derived growth factor C
422	W67577	CD74	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associat d)
423	NM_002996	CX3CL1	ch mokine (C-X3-C motif) ligand 1
424	AA024459		ESTs
425	NM_000163	GHR	growth hormone r ceptor

426	AA858162	NCAG1	NCAG1
427	BE327623		ESTs, Weakly similar to hypothetical protein FLJ20234 [Homo sapiens] [H.sapi ns]
428	BE671156	MAPRE2	microtubule-associated protein, RP/EB family, member 2
429	D12614	LTA	lymphotoxin alpha (TNF superfamily, member 1)
430	L13720	MGC5560	hypothetical protein MGC5560
431	U15131	ST5	suppression of tumorigenicity 5
432	Y00711	LDHB	lactate dehydrogenase B
433	AI651212		Homo sapiens cDNA FLJ31125 fis, clone IMR322000819.
434	M31159	IGFBP3	insulin-like growth factor binding protein 3
435	NM_014447	HSU52521	arfaptin 1
436	AB011089	TRIM2	tripartite motif-containing 2
437	BF969355	PDK4	pyruvate dehydrogenase kinase, isoenzyme 4
438	AK025950	KIAA1695	hypothetical protein FLJ22297
439	D86961	LHFPL2	lipoma HMGIC fusion partner-like 2
440	AK025953		Homo sapiens cDNA: FLJ22300 fis, clone HRC04759.
441	AJ223812	CALD1	caldesmon 1
442	R40594		Homo sapiens cDNA: FLJ22845 fis, clone KAIA5195.
443	AF145713	SCHIP1	schwannomin interacting protein 1
444	AK024966	FLJ21313	hypothetical protein FLJ21313
445	NM_005596	NFIB	nuclear factor I/B
446	NM_001613	ACTA2	actin, alpha 2, smooth muscle, aorta
447	H03641	FAM13A1	family with sequence similarity 13, member A1

Table 7 Genes commonly up-regulated in IDC

	ACCESSION	Symbol	TITLE
448	X14420	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)
449	AF044588	PRC1	protein regulator of cytokinesis 1
450	AF161499	HSPC150	HSPC150 protein similar to ubiquitin-conjugating enzyme
451	AA789233	COL1A1	collagen, type I, alpha 1
452	U16306	CSPG2	chondroitin sulfate proteoglycan 2 (versican)
453	NM_004425	ECM1	extracellular matrix protein 1
454	NM_006855	KDELR3	KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3
455	AI972071	CCNB1	cyclin B1
456	AF237709	TOPK	T-LAK cell-originated protein kinase
457	BE747327	HIST1H1C	histone 1, H1c
458	J03464	COL1A2	collagen, type I, alpha 2
459	AI080640	AGR2	anterior gradient 2 homolog (Xenepus laevis)
460	AA971042	RHPN1	rhophilin, Rho GTPase binding protein 1
461	AI419398	MGC33662	hypothetical protein MGC33662
462	AI149552		ESTs, Moderately similar to ERB2_HUMAN Receptor protein-tyrosin kinase erbB-2 precursor (p185erbB2) (NEU proto-oncogen) (C-erbB-2) (Tyrosine kinase-type c II surfac rec ptor HER2) (MLN 19) [H.sapi ns]
463	D14874	ADM	adrenom dullin

464	X03674	G6PD	glucose-6-phosphate dehydrogenase
465	NM_002358	MAD2L1	MAD2 mitotic arrest d ficient-lik 1 (yeast)
466	BF214508	CYCS	cytochrome c, somatic
467	BG030536	TOP2A	topoisomerase (DNA) II alpha 170kDa
468	X57766	MMP11	matrix metalloproteinase 11 (stromelysin 3)
469	AA029900	SULF1	sulfatase 1
470	AF053306	BUB1B	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)
471	AF074002	LGALS8	lectin, galactoside-binding, soluble, 8 (galectin 8)

Table 8 Genes commonly down-regulated in IDC

	ACCESSION	Symbol	TITLE
472	NM_004484	GPC3	glypican 3
473	NM_006219	PIK3CB	phosphoinositide-3-kinase, catalytic, beta polypeptide
474	BE793000	RBP1	retinol binding protein 1, cellular
475	AL117565	AXUD1	AXIN1 up-regulated 1
476	BF055342	ZNF6	zinc finger protein 6 (CMPX1)
477	U03688	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
478	AF038193		Homo sapiens, clone IMAGE:3610040, mRNA
479	X72760	LAMB2	laminin, beta 2 (laminin S)
480	J03817	GSTM1	glutathione S-transferase M1
481	M69226	MAOA	monoamine oxidase A
482	BF690180	WASF2	WAS protein family, member 2
483	AL133600	STAM2	signal transducing adaptor molecule (SH3 domain and ITAM motif) 2
484	AF215981	GPR2	G protein-coupled receptor 2
485	BG149764		Homo sapiens, clone IMAGE:5286091, mRNA, partial cds
486	AF067800	CLECSF6	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 6
487	AA713487	PIK3R1	phosphoinositide-3-kinase, regulatory subunit, polypeptide 1 (p85 alpha)
488	AA828505	FBXW7	F-box and WD-40 domain protein 7 (archipelago homolog, Drosophila)
489	AK021865	CKIP-1	CK2 interacting protein 1; HQ0024c protein
490	AK001605	FLJ10743	hypothetical protein FLJ10743
491	AI041186	HSPC182	HSPC182 protein
492	AA873363	ADH8	alcohol dehydrogenase 8
493	NM_013409	FST	follistatin
494	AK000322	FLJ20315	hypothetical protein FLJ20315
495	AB020637	KIAA0830	KIAA0830 protein
496	AA872040	INHBB	inhibin, beta B (activin AB beta polypeptide)
497	NM_004430	EGR3	early growth response 3
498	D59989		ESTs
499	D78013	DPYSL2	dihydropyrimidinas -like 2
500	AI081821		Homo sapiens mRNA; cDNA DKFZp313M0417 (from clon DKFZp313M0417)
501	AA309603	KIAA1430	KIAA1430 protein

502	NM_004107	FCGRT	Fc fragment of IgG, receptor, transporter, alpha
503	AW268719		Homo sapiens cDNA FLJ32438 fis, clone SKMUS2001402.
504	BF446578	LOC221002	CG4853 gene product
505	BG054844	ARHE	ras homolog gene family, member E
506	AF054987	ALDOC	aldolase C, fructose-bisphosphate
507	AI052390	FLJ20071	dymeclin
508	NM_004530	MMP2	matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)
509	AF054999	EPB41L2	erythrocyte membrane protein band 4.1-like 2
510	AU151591	NAV2	neuron navigator 2
511	AA447744		ESTs
512	R61253	ST6GalII	beta-galactoside alpha-2,6-sialyltransferase II

Table 9 Primer sequences for semi-quantitative RT-PCR experiments

ACCESSION	Symbol	Forward primer	Reverse primer
AI261804	EST	5'-CTGTTCTGGC TTCGTTATGT TCT-3' (SEQ ID NO:1)	5'-AGAAAATACG GTCCTCTTGT TGC-3' (SEQ ID NO:2)
AA205444	AP1S2	5'-CACTGTAATG CACGACATTT GA-3' (SEQ ID NO:3)	5'-GTTACAGCTT AGCACAAGGC ATC-3' (SEQ ID NO:4)
AA167194	LOC253782	5'-ACCTCTGAGT TTGATTTCCC AA-3' (SEQ ID NO:5)	5'-CGAGGCTTGT AACAATCTAC TGG-3' (SEQ ID NO:6)
AA676987	EST	5'-GAAACTGTAC GGGGGTTAAA GAG-3' (SEQ ID NO:7)	5'-CATCAATGTG GTGAGTGACA TCT-3' (SEQ ID NO:8)
H22566	DACH	5'-AAGCCCTTGG AACAGAACAT ACT-3' (SEQ ID NO:9)	5'-CAGTAAACGT GGTTCTCACA TTG-3' (SEQ ID NO:10)
NM_002046	GAPD	5'-CGACCACTTT GTCAAGCTCA-3' (SEQ ID NO:11)	5'-GGTTGAGCAC AGGGTACTTT ATT-3' (SEQ ID NO:12)

5

Table 10 List of genes with altered expression between well and poorly differentiated type in single case

	ACCESSION	Symbol	TITLE	p-value
513	AV729269	DKFZP564D166	putative ankyrin-repeat containing protein	3.1E-07
514	AI246554	NDUFA8	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8, 19kDa	1.4E-06
515	J04080	C1S	complement component 1, s subcomponent	1.4E-05
516	N93264	EST	Homo sapiens, clone IMAGE:4908933, mRNA	1.4E-05
517	NM_002318	LOXL2	lysyl oxidase-like 2	1.6E-05
518	J03464	COL1A2	collagen, type I, alpha 2	2.4E-05
519	U01184	FLII	flightless I homolog (Drosophila)	2.5E-05
520	X63556	FBN1	fibrillin 1 (Marfan syndrome)	3.8E-05
521	X78137	PCBP1	poly(rC) binding protein 1	4.6E-05
522	AK021534	EST	Homo sapiens cDNA FLJ11472 fis, clone HEMBA1001711.	6.3E-05
523	AK024012	NPD002	NPD002 protein	6.3E-05

524	AI200892	BIK	BCL2-interacting kill r (apoptosis-inducing)	9.1E-05
525	J03040	SPARC	s cr t d protein, acidic, cystein -rich (osteon ctin)	9.3E-05
526	AW970143	C6orf49	chromosome 6 open reading frame 49	1.0E-04
527	D62873	EST	Homo sapiens, clone IMAGE:5288080, mRNA	1.2E-04
528	D42041	G2AN	alpha glucosidase II alpha subunit	1.2E-04
529	AI376418	EST	Homo sapiens cDNA FLJ35169 fis, clone PLACE6012908.	1.7E-04
530	AK026744	FLJ23091	hypothetical protein FLJ23091	1.8E-04
531	AF026292	CCT7	chaperonin containing TCP1, subunit 7 (eta)	2.0E-04
532	Y10805	HRMT1L2	HMT1 hnRNP methyltransferase-like 2 (S. cerevisiae)	2.1E-04
533	L12350	THBS2	thrombospondin 2	2.1E-04
534	AK025706	AMPD2	adenosine monophosphate deaminase 2 (isoform L)	2.4E-04
535	BE618804	PIG11	p53-induced protein	2.5E-04
536	AV713686	RPS29	ribosomal protein S29	2.8E-04
537	M26481	TACSTD1	tumor-associated calcium signal transducer 1	2.8E-04
538	D00099	ATP1A1	ATPase, Na+/K+ transporting, alpha 1 polypeptide	2.9E-04
539	AA946602	ORMDL2	ORM1-like 2 (S. cerevisiae)	2.9E-04
540	NM_001533	HNRPL	heterogeneous nuclear ribonucleoprotein L	3.9E-04
541	BG107866	SIVA	CD27-binding (Siva) protein	4.4E-04
542	W72297	FLJ20533	hypothetical protein FLJ20533	4.4E-04
543	U76992	HTATSF1	HIV TAT specific factor 1	4.8E-04
544	AA191454	FIBP	fibroblast growth factor (acidic) intracellular binding protein	4.9E-04
545	BE903483	RPS20	ribosomal protein S20	5.4E-04
546	AJ005282	NPR2	natriuretic peptide receptor B/guanylate cyclase B (atrionatriuretic peptide receptor B)	5.5E-04
547	D86322	CLGN	calmegin	5.7E-04
548	AA621665	EST	EST	5.8E-04
549	M77349	TGFBI	transforming growth factor, beta-induced, 68kDa	6.3E-04
550	BE176466	ZAP3	ZAP3 protein	6.6E-04
551	AA776882	STMN4	stathmin-like 4	7.1E-04
552	AI261382	SH120	putative G-protein coupled receptor	7.1E-04
553	AB007618	COX7A2L	cytochrome c oxidase subunit VIIa polypeptide 2 like	7.2E-04
554	D21261	TAGLN2	transgelin 2	7.5E-04
555	M68864	LOC51035	ORF	7.7E-04
556	AB007836	TGFB1I1	transforming growth factor beta 1 induced transcript 1	8.1E-04
557	AA173339	EST	EST	8.4E-04
558	D87810	PMM1	phosphomannomutase 1	8.4E-04
559	M15798	ASNS	asparagine synthetase	8.7E-04
560	AW072418	B7	B7 protein	9.0E-04
561	D38293	AP3M2	adaptor-related protein complex 3, mu 2 subunit	9.5E-04
562	NM_018950	HLA-F	major histocompatibility complex, class I, F	1.0E-03
563	NM_001219	CALU	calum nin	1.1E-03
564	J04162	FCGR3A	Fc fragm nt of IgG, low affinity IIIa, r ceptor for (CD16)	1.1E-03
565	U09873	FSCN1	fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus)	1.1E-03

566	N51082	DACH	dachshund homolog (Drosophila)	1.3E-03
567	NM_004199	P4HA2	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylas), alpha polyp ptide II	1.3E-03
568	BE904196	GNB1	guanine nucleotide binding protein (G protein), beta polypeptide 1	1.3E-03
569	L08895	MEF2C	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)	1.3E-03
570	AK022670	C20orf6	chromosome 20 open reading frame 6	1.3E-03
571	AW157725	POLR2F	polymerase (RNA) II (DNA directed) polypeptide F	1.4E-03
572	NM_004939	DDX1	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 1	1.4E-03
573	X65463	RXRB	retinoid X receptor, beta	1.5E-03
574	Z68179	LY6E	lymphocyte antigen 6 complex, locus E	1.5E-03
575	BF976420	SNRPF	small nuclear ribonucleoprotein polypeptide F	1.5E-03
576	D79986	BTF	Bcl-2-associated transcription factor	1.5E-03
577	AK001023	NUBP2	nucleotide binding protein 2 (MinD homolog, E. coli)	1.6E-03
578	BE065329	EST	EST	1.6E-03
579	L34600	MTIF2	mitochondrial translational initiation factor 2	1.7E-03
580	D13630	BZW1	basic leucine zipper and W2 domains 1	1.7E-03
581	X15880	COL6A1	collagen, type VI, alpha 1	1.7E-03
582	AB003723	PIGQ	phosphatidylinositol glycan, class Q	1.7E-03
583	L36645	EPHA4	EphA4	1.7E-03
584	BF974358	RPS27	ribosomal protein S27 (metallopanstimulin 1)	1.8E-03
585	AA747449	HIP2	huntingtin interacting protein 2	1.9E-03
586	AA283813	FLJ12150	hypothetical protein FLJ12150	2.0E-03
587	L38995	TUFM	Tu translation elongation factor, mitochondrial	2.0E-03
588	N67293	EST	Homo sapiens cDNA FLJ11997 fis, clone HEMBB1001458.	2.1E-03
589	AB014549	KIAA0649	KIAA0649 gene product	2.1E-03
590	D38305	TOB1	transducer of ERBB2, 1	2.2E-03
591	L40391	TMP21	transmembrane trafficking protein	2.2E-03
592	H28960	EST	ESTs	2.2E-03
593	U86753	CDC5L	CDC5 cell division cycle 5-like (S. pombe)	2.3E-03
594	AI143226	BLP1	BBP-like protein 1	2.3E-03
595	M57730	EFNA1	ephrin-A1	2.3E-03
596	AI928868	UBR1	ubiquitin protein ligase E3 component n-recognin 1	2.3E-03
597	AF077044	RPAC2	likely ortholog of mouse RNA polymerase 1-3 (16 kDa subunit)	2.3E-03
598	AF097431	LEPRE1	leucine proline-enriched proteoglycan (leprecan) 1	2.4E-03
599	NM_004350	RUNX3	runt-related transcription factor 3	2.4E-03
600	AL162047	NCOA4	nuclear receptor coactivator 4	2.5E-03
601	BF915013	EST	Homo sapiens cDNA FLJ37302 fis, clone BRAMY2016009.	2.5E-03
602	Z37166	BAT1	HLA-B associated transcript 1	2.5E-03
603	M81349	SAA4	s rum amyloid A4, constitutive	2.6E-03
604	AL137338	SEC63L	SEC63 prot in	2.6E-03
605	AI745624	ELL2	ELL-relat d RNA polymerase II, longation factor	2.6E-03
606	BG167522	HSPC016	hypothetical protein HSPC016	2.6E-03

607	U58766	TSTA3	tissue specific transplantation antigen P35B	2.7E-03
608	J04474	BCKDHA	branched chain keto acid dehydrogenas E1, alpha polypeptid (maple syrup urin dis ase)	2.7E-03
609	H15977	EST	Homo sapiens cDNA FLJ30781 fis, clone FEBRA2000874.	2.8E-03
610	AL049339	CPD	carboxypeptidase D	2.8E-03
611	AL133555	C20orf108	chromosome 20 open reading frame 108	2.9E-03
612	AW662518	FLJ10876	hypothetical protein FLJ10876	2.9E-03
613	BE883507	CGGBP1	CGG triplet repeat binding protein 1	2.9E-03
614	BE797472	RPL17	ribosomal protein L17	3.0E-03
615	U41371	SF3B2	splicing factor 3b, subunit 2, 145kDa	3.0E-03
616	L39068	DHPS	deoxyhypusine synthase	3.1E-03
617	NM_004517	ILK	integrin-linked kinase	3.1E-03
618	U14972	RPS10	ribosomal protein S10	3.2E-03
619	U61500	TMEM1	transmembrane protein 1	3.3E-03
620	NM_002719	PPP2R5C	protein phosphatase 2, regulatory subunit B (B56), gamma isoform	3.3E-03
621	AF053233	VAMP8	vesicle-associated membrane protein 8 (endobrevin)	3.3E-03
622	NM_002822	PTK9	PTK9 protein tyrosine kinase 9	3.3E-03
623	U16996	DUSP5	dual specificity phosphatase 5	3.3E-03
624	AV705747	SFRS7	splicing factor, arginine/serine-rich 7, 35kDa	3.3E-03
625	AF178984	IER5	immediate early response 5	3.3E-03
626	Z29093	DDR1	discoidin domain receptor family, member 1	3.3E-03
627	AB024536	ISLR	immunoglobulin superfamily containing leucine-rich repeat	3.3E-03
628	BF791601	EMP2	epithelial membrane protein 2	3.3E-03
629	AF061737	SPC18	signal peptidase complex (18kD)	3.3E-03
630	AB002386	EZH1	enhancer of zeste homolog 1 (Drosophila)	3.5E-03
631	AA634090	EST	Homo sapiens, Similar to heterogeneous nuclear ribonucleoprotein A1, clone IMAGE:2900557, mRNA	3.5E-03
632	AK023674	FLJ13612	likely ortholog of neuronally expressed calcium binding protein	3.6E-03
633	D13626	GPR105	G protein-coupled receptor 105	3.7E-03
634	AK026849	TSPYL	TSPY-like	3.8E-03
635	Y18643	METTL1	methyltransferase-like 1	3.9E-03
636	AF176699	FBXL4	F-box and leucine-rich repeat protein 4	3.9E-03
637	NM_003977	AIP	aryl hydrocarbon receptor interacting protein	3.9E-03
638	AK000498	HARS	histidyl-tRNA synthetase	4.0E-03
639	U05237	FALZ	fetal Alzheimer antigen	4.0E-03
640	BF696304	FLJ14735	hypothetical protein FLJ14735	4.0E-03
641	X14420	COL3A1	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	4.1E-03
642	BE796098	NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductas)	4.3E-03
643	X60221	ATP5F1	ATP synthase, H+ transporting, mitochondrial F0 compl x, subunit b, isoform 1	4.4E-03
644	AA135341	GCN5L2	GCN5 general control of amino-acid synth sis 5-lik 2	4.6E-03

			(yeast)	
645	AF009368	CREB3	cAMP r sponsive elem nt binding prot in 3 (luman)	4.7E-03
646	BF970013	SPC12	signal peptidas 12kDa	4.7E-03
647	W45522	ATPIF1	ATPase inhibitory factor 1	4.7E-03
648	AI733356	EST	Homo sapiens cDNA FLJ31746 fis, clone NT2RI2007334.	4.8E-03
649	AW117927	EIF3S9	eukaryotic translation initiation factor 3, subunit 9 eta, 116kDa	4.8E-03
650	AF275798	CCT5	chaperonin containing TCP1, subunit 5 (epsilon)	5.0E-03
651	AI937126	WTAP	Wilms' tumour 1-associating protein	5.0E-03
652	AK024891	LOC253782	hypothetical protein LOC253782	5.1E-03
653	D13629	KTN1	kinectin 1 (kinesin receptor)	5.2E-03
654	AI682994	AHCYL1	S-adenosylhomocysteine hydrolase-like 1	5.3E-03
655	BF980325	ATP6V1C2	ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C isoform 2	5.3E-03
656	AI378996	NCL	nucleolin	5.3E-03
657	D88153	HYA22	HYA22 protein	5.3E-03
658	S67310	BF	B-factor, properdin	5.4E-03
659	AW438585	EST	Homo sapiens, clone IMAGE:5273745, mRNA	5.4E-03
660	M12267	OAT	ornithine aminotransferase (gyrate atrophy)	5.5E-03
661	AB001636	DDX15	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 15	5.7E-03
662	D13315	GLO1	glyoxalase I	5.9E-03
663	AF244931	WDR10	WD repeat domain 10	5.9E-03
664	AL050094	IDH3B	isocitrate dehydrogenase 3 (NAD+) beta	6.0E-03
665	AK022881	KIAA1272	KIAA1272 protein	6.0E-03
666	AI720096	RPL29	ribosomal protein L29	6.1E-03
667	Y12781	TBL1X	transducin (beta)-like 1X-linked	6.2E-03
668	AI014538	LOC92170	hypothetical protein BC004409	6.2E-03
669	NM_020987	ANK3	ankyrin 3, node of Ranvier (ankyrin G)	6.3E-03
670	NM_004387	NKX2-5	NK2 transcription factor related, locus 5 (Drosophila)	6.3E-03
671	J03817	GSTM1	glutathione S-transferase M1	6.3E-03
672	BF435769	EST	ESTs, Weakly similar to hypothetical protein FLJ20378 [Homo sapiens] [H.sapiens]	6.5E-03
673	AL390147	DKFZp547D065	hypothetical protein DKFZp547D065	6.5E-03
674	AA961412	UBA52	ubiquitin A-52 residue ribosomal protein fusion product 1	6.6E-03
675	NM_002702	POU6F1	POU domain, class 6, transcription factor 1	6.6E-03
676	M58050	MCP	membrane cofactor protein (CD46, trophoblast-lymphocyte cross-reactive antigen)	6.6E-03
677	NM_001293	CLNS1A	chloride channel, nucleotide-sensitive, 1A	6.7E-03
678	BF213049	COX7A2	cytochrome c oxidase subunit VIIa polypeptide 2 (liver)	6.7E-03
679	AF236056	GOLPH2	golgi phosphoprotein 2	6.7E-03
680	U79285	NMT1	N-myristoyltransferase 1	6.8E-03
681	AB027196	RNF10	ring fing r protein 10	6.9E-03
682	AA036952	FLJ30973	hypothetical protein FLJ30973	7.0E-03

683	AW732157	TOP1MT	mitochondrial topoisom rase I	7.1E-03
684	AL049319	FLJ14547	hypothetical protein FLJ14547	7.3E-03
685	BE613161	EST	Homo sapiens cDNA FLJ37042 fis, clone BRACE2011947.	7.3E-03
686	U28749	HMGA2	high mobility group AT-hook 2	7.3E-03
687	BF793677	MGC49942	hypothetical protein MGC49942	7.4E-03
688	BG032216	FLJ20287	hypothetical protein FLJ20287	7.4E-03
689	AL449244	PP2447	hypothetical protein PP2447	7.5E-03
690	AK024103	EST	Homo sapiens cDNA FLJ14041 fis, clone HEMBA1005780.	7.5E-03
691	U17838	PRDM2	PR domain containing 2, with ZNF domain	7.5E-03
692	D86479	AEBP1	AE binding protein 1	7.5E-03
693	D50420	NHP2L1	NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae)	7.5E-03
694	D87258	PRSS11	protease, serine, 11 (IGF binding)	7.5E-03
695	BF434108	HSPC171	HSPC171 protein	7.6E-03
696	NM_000705	ATP4B	ATPase, H+/K+ exchanging, beta polypeptide	7.7E-03
697	AF077599	SBB103	hypothetical SBBI03 protein	7.7E-03
698	NM_001530	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	7.8E-03
699	AB023204	EPB41L3	erythrocyte membrane protein band 4.1-like 3	7.8E-03
700	AA253194	PIGPC1	p53-induced protein PIGPC1	7.9E-03
701	BE502341	C17orf26	chromosome 17 open reading frame 26	7.9E-03
702	AL050265	TARDBP	TAR DNA binding protein	8.0E-03
703	AK001643	FLJ10781	hypothetical protein FLJ10781	8.3E-03
704	BG179412	COX7B	cytochrome c oxidase subunit VIIb	8.6E-03
705	X03212	KRT7	keratin 7	8.8E-03
706	L07033	HMGCL	3-hydroxymethyl-3-methylglutaryl-Coenzyme A lyase (hydroxymethylglutaricaciduria)	9.0E-03
707	M19383	ANXA4	annexin A4	9.0E-03
708	NM_001273	CHD4	chromodomain helicase DNA binding protein 4	9.1E-03
709	NM_004461	FARSL	phenylalanine-tRNA synthetase-like	9.1E-03
710	AI192880	CD44	CD44 antigen (homing function and Indian blood group system)	9.1E-03
711	AF038961	MPDU1	mannose-P-dolichol utilization defect 1	9.5E-03
712	U67322	C20orf18	chromosome 20 open reading frame 18	9.5E-03
713	AA521017	EST	EST	9.5E-03
714	AA811043	RNASE6PL	ribonuclease 6 precursor	9.9E-03
715	AA536113	TMEPAI	transmembrane, prostate androgen induced RNA	9.9E-03
716	BF973104	LOC201725	hypothetical protein LOC201725	9.9E-03
717	NM_000293	PHKB	phosphorylase kinase, beta	9.9E-03
718	NM_000548	TSC2	tuberous sclerosis 2	1.0E-02

Tabl 11 List of gen s with altered expression between node-positive and node-negative tumors

	ACCESSION	Symbol	TITLE	P-value	+ or -
719	BF686125	UBA52	ubiquitin A-52 residue ribosomal protein fusion product 1	8.1E-09	-

720	AA634090		Homo sapiens, Similar to heterogen ous nuclear ribonucleoprot in A1, clone IMAGE:2900557, mRNA	1.4E-07	-
721	L00692	CEACAM3	carcinoembryonic antigen-related cell adhesion molecule 3	4.2E-07	-
722	AW954403	VAMP3	vesicle-associated membrane protein 3 (cellubrevin)	2.2E-06	+
723	AA865619	C21orf97	chromosome 21 open reading frame 97	2.6E-06	-
724	W74502	MGC11257	hypothetical protein MGC11257	2.4E-05	+
725	NM_002094	GSPT1	G1 to S phase transition 1	2.7E-05	+
726	T55178	KIAA1040	KIAA1040 protein	3.2E-05	-
727	L36983	DNM2	dynamin 2	4.1E-05	+
728	Z21507	EEF1D	eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	5.2E-05	-
729	AI581728	CFL1	cofilin 1 (non-muscle)	8.0E-05	+
730	NM_001293	CLNS1A	chloride channel, nucleotide-sensitive, 1A	9.0E-05	+
731	BF680847	SENP2	sentrin-specific protease	9.0E-05	+
732	AF100743	NDUFS3	NADH dehydrogenase (ubiquinone) Fe-S protein 3, 30kDa (NADH-coenzyme Q reductase)	9.8E-05	+
733	NM_004960	FUS	fusion, derived from t(12;16) malignant liposarcoma	9.8E-05	-
734	AK023975	NOP5/NOP58	nucleolar protein NOP5/NOP58	1.3E-04	+
735	AF083245	PSMD13	proteasome (prosome, macropain) 26S subunit, non-ATPase, 13	1.5E-04	+
736	AA129776	SUOX	sulfite oxidase	1.8E-04	+
737	U55766	HRB2	HIV-1 rev binding protein 2	2.0E-04	+
738	BF526092	LOC154467	hypothetical protein BC003515	2.1E-04	+
739	BF677579	THTPA	thiamine triphosphatase	2.3E-04	+
740	X98260	ZRF1	zuotin related factor 1	2.3E-04	+
741	BE440010	LOC51255	hypothetical protein LOC51255	2.7E-04	+
742	AF007165	DEAF1	deformed epidermal autoregulatory factor 1 (Drosophila)	2.7E-04	+
743	X78687	NEU1	sialidase 1 (lysosomal sialidase)	3.0E-04	+
744	AW965200		Homo sapiens, clone IMAGE:5286019, mRNA	3.1E-04	-
745	AK023240	UGCGL1	UDP-glucose ceramide glucosyltransferase-like 1	3.1E-04	+
746	M95712	BRAF	v-raf murine sarcoma viral oncogene homolog B1	3.7E-04	+
747	L38995	TUFM	Tu translation elongation factor, mitochondrial	3.9E-04	+
748	AW014268	FLJ10726	hypothetical protein FLJ10726	4.2E-04	+
749	D49547	DNAJB1	DnaJ (Hsp40) homolog, subfmaily B, member 1	4.4E-04	+
750	BE466450	AP4S1	adaptor-related protein complex 4, sigma 1 subunit	4.5E-04	+
751	AB007944	KIAA0475	KIAA0475 gen product	4.9E-04	-
752	AF034091	MRPL40	mitochondrial ribosomal protein L40	5.1E-04	+

Table12 Histoclinical information

ID	age in operation	menopause status	T	N	M	Stag	Histrogical type	Lymphocytic infiltrate	Angioinvasion	ER	PgR
MMK010003	51	pre	2	1	0	2	a3	3	0	+	+
MMK010004	47	pre	2	1	0	2	a1	0	0	+	+
MMK010005	44	pre	2	0	0	2	a1	1	0	+	+
MMK010013	45	pre	2	1	0	2	a1	1	0	−	−
MMK010016	44	pre	2	0	0	2	a2	0	0	−	−
MMK010025	46	pre	2	0	0	2	a1	0	0	+	+
MMK010031	29	pre	2	2	0	3	a3	3	0	−	−
MMK010037	62	post	0	0	0	0	Ia	0	0	+	+
MMK010042	47	pre	2	1	0	2	a3	1	2	+	+
MMK010086	42	pre	2	0	0	2	a1	0	0	+	+
MMK010102	51	pre	2	1	0	3	a2	3	0	+	+
MMK010110	39	pre	2	0	0	2	a1	2	0	−	−
MMK010129	52	pre	2	2	0	3	a1	2	0	−	−
MMK010135	41	pre	2	0	0	2	a1	0	0	+	+
MMK010138	38	pre	2	0	0	2	a1	0	0	+	+
MMK010145	51	pre	2	1	0	2	a3	0	0	+	+
MMK010147	49	pre	2	1	0	2	a1	1	0	+	+
MMK010149	35	pre	2	0	0	2	a3	1	0	−	−
MMK010175	38	pre	2	0	0	2	a3	0	0	+	+
MMK010178	51	pre	0	0	0	0	Ia	0	0	+	+
MMK010207	40	pre	2	0	0	2	a1	0	0	+	+
MMK010214	42	pre	2	1	0	2	a1	0	0	−	−
MMK010247	48	pre	2	1	0	2	a2	3	0	−	−
MMK010252	52	pre	2	1	0	2	a2	0	0	−	−
MMK010255	47	pre	2	0	0	2	a2	0	0	−	−
MMK010302	46	pre	2	1	0	2	a2	2	1	−	−
MMK010304	48	pre	2	1	0	2	a3	1	0	+	+
MMK010326	53	post	0	0	0	0	Ia	0	0	−	−
MMK010327	43	pre	2	1	0	2	a1	1	1	+	+
MMK010341	42	pre	2	1	0	2	a1	2	0	+	+
MMK010370	46	pre	2	1	0	2	a3	2	0	+	+
MMK010397	38	pre	2	1	0	2	a3	3	2	+	+
MMK010411	46	pre	2	0	0	2	a1	0	0	+	+
MMK010431	50	pre	2	0	0	2	a3	0	0	−	−
MMK010435	49	pre	2	1	0	2	a3	0	0	+	+
MMK010453	49	pre	2	1	0	2	a3	3	0	+	+
MMK010471	42	pre	2	1	0	2	a1	3	0	−	−
MMK010473	40	pr	2	1	0	2	a2	0	0	−	−
MMK010478	38	pr	2	2	0	3	a2	0	0	+	+
MMK010491	46	pr	2	0	0	2	a3	1	0	+	+
MMK010497	44	pre	0	0	0	0	Ia	0	0	−	+

MMK010500	45	pr	2	0	0	2	a1	0	0	+	+
MMK010502	51	pre	2	0	0	2	a2	0	0	–	–
MMK010508	51	pre	2	1	0	2	a2	0	0	–	–
MMK010521	21	pre	2	0	0	2	a1	1	1	–	–
MMK010552	49	pre	2	0	0	2	a2	0	0	–	–
MMK010554	51	pre	2	0	0	2	a3	2	0	+	+
MMK010571	45	pre	2	1	1	4	a3	3	0	+	+
MMK010591	40	pre	0	0	0	0	Ia	0	0	–	+
MMK010613	37	pre	0	0	0	0	Ia	0	0	–	+
MMK010623	39	pre	2	1	0	2	a1	3	0	+	+
MMK010624	39	pre	2	1	0	2	a1	3	0	+	+
MMK010626	48	pre	2	0	0	2	a1	1	1	–	–
MMK010631	41	pre	2	0	0	2	a1	0	0	+	+
MMK010640	35	pre	0	0	0	0	Ia	0	0	+	+
MMK010644	47	pre	2	2	0	2	a3	3	0	+	+
MMK010646	37	pre	2	1	0	2	a3	1	0	+	+
MMK010660	46	pre	2	0	0	2	a1	0	0	–	–
MMK010671	45	pre	2	0	0	2	a1	0	0	–	–
MMK010679	68	post	0	0	0	0	Ia	0	0	+	+
MMK010680	58	post	0	0	0	0	Ia	0	0	–	+
MMK010709	33	pre	2	0	0	2	a3	0	2	–	–
MMK010711	51	pre	0	0	0	0	Ia	0	0	–	+
MMK010724	40	pre	2	1	0	2	a3	3	2	+	+
MMK010744	41	pre	0	0	0	0	Ia	0	0	+	+
MMK010758	40	pre	2	1	0	2	a1	0	1	+	+
MMK010760	42	pre	2	0	0	2	a1	0	0	+	+
MMK010762	50	pre	2	1	0	2	a3	3	1	+	+
MMK010769	33	pre	2	0	0	2	a2	0	0	–	–
MMK010772	45	pre	2	1	0	2	a3	2	0	–	–
MMK010779	46	pre	2	1	0	2	a2	0	1	–	–
MMK010780	31	pre	2	0	0	2	a2	0	0	–	–
MMK010781	44	pre	2	0	0	2	a3	0	2	+	+
MMK010794	52	pre	2	1	0	2	a3	2	1	+	+
MMK010818	51	pre	2	0	0	2	a1	0	2	+	+
MMK010835	42	pre	0	0	0	0	Ia	0	0	+	+
MMK010846	47	pre	2	0	0	2	a1	0	0	+	+
MMK010858	42	pre	2	1	0	2	a3	2	3	+	+
MMK010864	52	pre	2	1	0	2	a1	0	1	–	–
MMK010869	45	pre	2	0	0	2	a1	0	1	–	–
MMK010903	47	pre	2	0	0	2	a1	0	0	+	+

Industrial Applicability

The gene-expression analysis of breast cancer described herein, obtained through a combination of laser-capture dissection and genome-wide cDNA microarray, has identified

specific genes as targets for cancer prevention and therapy. Based on the expression of a subset of these differentially expressed genes, the present invention provides molecular diagnostic markers for identifying or detecting breast cancer.

The methods described herein are also useful in the identification of additional molecular targets for prevention, diagnosis and treatment of breast cancer. The data reported herein add to a comprehensive understanding of breast cancer, facilitate development of novel diagnostic strategies, and provide clues for identification of molecular targets for therapeutic drugs and preventative agents. Such information contributes to a more profound understanding of breast tumorigenesis, and provide indicators for developing novel strategies for diagnosis, treatment, and ultimately prevention of breast cancer.

All patents, patent applications, and publications cited herein are incorporated by reference in their entirety. Furthermore, while the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.

CLAIMS

1. A method of diagnosing breast cancer or a predisposition to developing breast cancer in a subject, comprising determining a level of expression of a breast cancer-associated gene in a patient derived biological sample, wherein an increase or decrease of said level compared to a normal control level of said gene indicates that said subject suffers from or is at risk of developing breast cancer.

2. The method of claim 1, wherein said breast cancer-associated gene is selected from the group consisting of the genes listed in table 3, 5 and 7, wherein an increase in said level compared to a normal control level indicates said subject suffers from or is at risk of developing breast cancer.

3. The method of claim 2, wherein said increase is at least 10% greater than said normal control level.

4. The method of claim 1, wherein said breast cancer-associated gene is selected from the group consisting of the genes listed in table 4, 6 and 8, wherein a decrease in said level compared to a normal control level indicates said subject suffers from or is at risk of developing breast cancer.

5. The method of claim 4, wherein said decrease is at least 10% lower than said normal control level.

6. A method of claim 1, wherein said breast cancer is IDC.

7. The method of claim 6, wherein said breast cancer-associated gene is selected from the group consisting of the genes listed in table 7, wherein an increase in said level compared to a normal control level indicates said subject suffers from or is at risk of developing IDC.

8. The method of claim 7, wherein said increase is at least 10% greater than said normal control level.

9. The method of claim 6, wherein said breast cancer-associated gene is selected from the group consisting of the genes listed in table 8, wherein a decrease in said level compared to a normal control level indicates said subject suffers from or is at risk of developing IDC.

10. The method of claim 9, wherein said decrease is at least 10% lower than said normal control level.

11. The method of claim 1, wherein said method further comprises determining said level of expression of a plurality of breast cancer-associated genes.

12. The method of claim 1, wherein the expression level is determined by any one method select from the group consisting of:
(a) detecting the mRNA of the breast cancer-associated genes,
(b) detecting the protein encoded by the breast cancer-associated genes, and
(c) detecting the biological activity of the protein encoded by the breast cancer-associated genes.

13 The method of claim 12, wherein said detection is carried out on a DNA array.

14 The method of claim 1, wherein said biological sample comprises an epithelial cell.

15 The method of claim 1, wherein said biological sample comprises a breast cancer cell.

16 The method of claim 1 wherein said biological sample comprises an epithelial cell from a breast cancer cell.

17 A breast cancer reference expression profile, comprising a pattern of gene expression of two or more genes selected from the group consisting of the genes listed in table 3, 4, 5, 6, 7 or 8.

18 A breast cancer reference expression profile, comprising a pattern of gene expression of two or more genes selected from the group consisting of the genes listed in table 3, 5 and 7.

19 A breast cancer reference expression profile, comprising a pattern of gene expression of two or more genes selected from the group consisting of the genes listed in table 4, 6 and 8.

20 A method of screening for a compound for treating or preventing breast cancer, said method comprising the steps of:
a) contacting a test compound with a polypeptide encoded by a polynucleotide selected from the group consisting of the genes listed in table 3, 4, 5, 6, 7 or 8;
b) detecting the binding activity between the polypeptide and the test compound; and

c) selecting a compound that binds to the polypeptide.

21. A method of screening for a compound for treating or preventing breast cancer, said method comprising the steps of:

a) contacting a candidate compound with a cell expressing one or more marker genes, wherein the one or more marker genes is selected from the group consisting of the genes listed in table 3, 4, 5, 6, 7 or 8; and

b) selecting a compound that reduces the expression level of one or more marker genes selected from the group consisting of the genes listed in table 3, 5, and 7, or elevates the expression level of one or more marker genes selected from the group consisting of the genes listed in table 4, 6 and 8.

22. The method of claim 21, wherein said cell comprises a breast cancer cell.

23. A method of screening for a compound for treating or preventing breast cancer, said method comprising the steps of:

a) contacting a test compound with a polypeptide encoded by a polynucleotide selected from the group consisting of the genes listed in table 3, 4, 5, 6, 7 or 8;

b) detecting the biological activity of the polypeptide of step (a); and

c) selecting a compound that suppresses the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of the genes listed in table 3, 5 and 7 in comparison with the biological activity detected in the absence of the test compound, or enhances the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of the genes listed in table 4, 6 and 8 in comparison with the biological activity detected in the absence of the test compound.

24. A method of screening for compound for treating or preventing breast cancer, said method comprising the steps of:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of one or more marker genes and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced, wherein the one or more marker genes are selected from the group consisting of the genes listed in table 3, 4, 5, 6, 7 or 8

b) measuring the activity of said reporter gene; and

c) selecting a compound that reduces the expression level of said reporter gene when said marker gene is an up-regulated marker gene selected from the group consisting of the genes listed in table 3, 5 and 7, or that enhances the expression level of said reporter gene when said marker gene is a down-regulated marker gene selected from the group consisting of the genes listed in table 4, 6 and 8, as compared to a control.

25 A method of claim 20, wherein said breast cancer is IDC, said method comprising the steps of:

a) contacting a test compound with a polypeptide encoded by a polynucleotide selected from the group consisting of the genes listed in table 7 or 8;

b) detecting the binding activity between the polypeptide and the test compound; and

c) selecting a compound that binds to the polypeptide.

26. A method of claim 21, wherein said breast cancer is IDC, said method comprising the steps of:

a) contacting a candidate compound with a cell expressing one or more marker genes, wherein the one or more marker genes is selected from the group consisting of the genes listed in table 7 or 8; and

b) selecting a compound that reduces the expression level of one or more marker genes selected from the group consisting of the genes listed in table 7, or elevates the expression level of one or more marker genes selected from the group consisting of the genes listed in table 8.

27. The method of claim 26, wherein said cell comprises a IDC cell.

28. A method of claim 23, wherein said breast cancer is IDC, said method comprising the steps of:

a) contacting a test compound with a polypeptide encoded by a polynucleotide selected from the group consisting of the genes listed in table 7 or 8;

b) detecting the biological activity of the polypeptide of step (a); and

c) selecting a compound that suppresses the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of the genes listed in table 7 in comparison with the biological activity detected in the absence of the test compound, or enhances the biological activity of the polypeptide encoded by

the polynucleotide selected from the group consisting of the genes listed in table 8 in comparison with the biological activity detected in the absence of the test compound.

29. A method of claim 24, wherein said breast cancer is IDC, said method comprising the steps of:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of one or more marker genes and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced, wherein the one or more marker genes are selected from the group consisting of the genes listed in table 7 or 8

b) measuring the activity of said reporter gene; and

c) selecting a compound that reduces the expression level of said reporter gene when said marker gene is an up-regulated marker gene selected from the group consisting of the genes listed in table 7, or that enhances the expression level of said reporter gene when said marker gene is a down-regulated marker gene selected from the group consisting of the genes listed in table 8, as compared to a control.

30. A kit comprising a detection reagent which binds to two or more nucleic acid sequences selected from the group consisting of the genes listed in table 3, 4, 5, 6, 7 or 8 or polypeptides encoded thereby.

31. An array comprising two or more nucleic acids which bind to one or more nucleic acid sequences selected from the group consisting of the genes listed in table 3, 4, 5, 6, 7 or 8.

32. A method of treating or preventing breast cancer in a subject comprising administering to said subject an antisense composition, said composition comprising a nucleotide sequence complementary to a coding sequence selected from the group consisting of the genes listed in table 3, 5 and 7.

33. A method of treating or preventing breast cancer in a subject comprising administering to said subject a siRNA composition, wherein said composition reduces the expression of a nucleic acid sequence selected from the group consisting of the genes listed in table 3, 5 and 7.

34. A method for treating or preventing breast cancer in a subject comprising the step of administering to said subject a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by any one gene selected from the group consisting of the genes listed in table 3, 5 and 7.

35. A method of treating or preventing breast cancer in a subject comprising administering to said subject a vaccine comprising a polypeptide encoded by a nucleic acid selected from the group consisting of the genes listed in table 3, 5 and 7 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide.

36. A method of treating or preventing breast cancer in a subject comprising administering to said subject a compoud that increases the expression or activity of a polynucleotide selected from the group consisting of the genes listed in table 4, 6 and 8

37. A method for treating or preventing breast cancer in a subject, said method comprising the step of administering a compound that is obtained by the method according to any one of claims 20-24.

38. A method of treating or preventing breast cancer in a subject comprising administering to said subject a pharmaceutically effective amount of a polynucleotide select from the group consisting of the genes listed in table 4, 6 and 8, or polypeptide encoded by thereof.

39. A method of claim 32, wherein said breast cancer is IDC, said antisence composition comprises a nucleotide sequence complementary to a coding sequence selected from the group consisting of the genes listed in table 7.

40. A method of claim 33, wherein said breast cancer is IDC, said siRNA composition reduces the expression of a nucleic acid sequence selected from the group consisting of the genes listed in table 7.

41. A method of claim 34, wherein said breast cancer is IDC, said antibody or fragment thereof binds to a protein encoded by any one gene selected from the group consisting of the genes listed in table 7.

42. A method of claim 35, wherein said breast cancer is IDC, said vaccine comprises a polypeptide encoded by a nucleic acid selected from the group consisting of the genes listed in table 7 or an immunologically active fragment of said polypeptide, or a

polynucleotide encoding the polypeptide.

43. A method of claim 36, wherein said breast cancer is IDC, said compound increases the expression or activity of a polynucleotide selected from the group consisting of the genes listed in table 8

44. A method of claim 37, wherein said breast cancer is IDC, said compound is obtained by the method according to any one of claims 25-29.

45. A method of claim 38, wherein said breast cancer is IDC, said polynucleotide is selected from the group consisting of the genes listed in table 8 or polypeptide encoded by thereof.

46. A composition for treating or preventing breast cancer, said composition comprising a pharmaceutically effective amount of an antisense polynucleotide or small interfering RNA against a polynucleotide select from the group consisting of the genes listed in table 3, 5 and 7.

47. A composition for treating or preventing breast cancer, said composition comprising a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by any one gene selected from the group consisting of the genes listed in table 3, 5 and 7.

48. A composition for treating or preventing breast cancer, said composition comprising a pharmaceutically effective amount of the compound selected by the method of any one of claims 20-24 as an active ingredient, and a pharmaceutically acceptable carrier.

49. A composition of claim 46, wherein said breast cancer is IDC, said polynucleotide is select from the group consisting of the genes listed in table 7.

50. A composition of claim 47, wherein said breast cancer is IDC, said protein is encoded by any one gene selected from the group consisting of the genes listed in table 7.

51. A composition of claim 48, wherein said breast cancer is IDC, said compound is selected by the method of any one of claims 25-29.

52. A method of screening for a compound for treating or preventing invasion of breast cancer, said method comprising the steps of:

a) contacting a test compound with a polypeptide encoded by a polynucleotide selected

from the group consisting of the genes listed in table 5 or 6;

b) detecting the binding activity between the polypeptide and the test compound; and

c) selecting a compound that binds to the polypeptide.

53. A method of screening for a compound for treating or preventing invasion of breast cancer, said method comprising the steps of:

a) contacting a candidate compound with a cell expressing one or more marker genes, wherein the one or more marker genes is selected from the group consisting of the genes listed in table 5 or 6; and

b) selecting a compound that reduces the expression level of one or more marker genes selected from the group consisting of the genes listed in table 5, or elevates the expression level of one or more marker genes selected from the group consisting of the genes listed in table 6.

54. The method of claim 53, wherein said cell comprises a breast cancer cell.

55. A method of screening for a compound for treating or preventing invasion of breast cancer, said method comprising the steps of:

a) contacting a test compound with a polypeptide encoded by a polynucleotide selected from the group consisting of the genes listed in table 5 or 6;

b) detecting the biological activity of the polypeptide of step (a); and

c) selecting a compound that suppresses the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of the genes listed in table 5 in comparison with the biological activity detected in the absence of the test compound, or enhances the biological activity of the polypeptide encoded by the polynucleotide selected from the group consisting of the genes listed in table 6 in comparison with the biological activity detected in the absence of the test compound.

56. A method of screening for compound for treating or preventing invasion of breast cancer, said method comprising the steps of:

a) contacting a candidate compound with a cell into which a vector comprising the transcriptional regulatory region of one or more marker genes and a reporter gene that is expressed under the control of the transcriptional regulatory region has been

introduced, wherein the one or more marker genes are selected from the group consisting of the genes listed in table 5 or 6

b) measuring the activity of said reporter gene; and

c) selecting a compound that reduces the expression level of said reporter gene when said marker gene is an up-regulated marker gene selected from the group consisting of the genes listed in table 5, or that enhances the expression level of said reporter gene when said marker gene is a down-regulated marker gene selected from the group consisting of the genes listed in table 6, as compared to a control.

57. A method of treating or preventing invasion of breast cancer in a subject comprising administering to said subject an antisense composition, said composition comprising a nucleotide sequence complementary to a coding sequence selected from the group consisting of the genes listed in table 5.

58. A method of treating or preventing invasion of breast cancer in a subject comprising administering to said subject a siRNA composition, wherein said composition reduces the expression of a nucleic acid sequence selected from the group consisting of the genes listed in table 5.

59. A method for treating or preventing invasion of breast cancer in a subject comprising the step of administering to said subject a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by any one gene selected from the group consisting of the genes listed in table 5.

60. A method of treating or preventing invasion of breast cancer in a subject comprising administering to said subject a vaccine comprising a polypeptide encoded by a nucleic acid selected from the group consisting of the genes listed in table 5 or an immunologically active fragment of said polypeptide, or a polynucleotide encoding the polypeptide.

61. A method of treating or preventing invasion of breast cancer in a subject comprising administering to said subject a compoud that increases the expression or activity of a polynucleotide selected from the group consisting of the genes listed in table 6

62. A method for treating or preventing invasion of breast cancer in a subject, said method comprising the step of administering a compound that is obtained by the method

according to any one of claims 52-56.

63. A method of treating or preventing invasion of breast cancer in a subject comprising administering to said subject a pharmaceutically effective amount of a polynucleotide select from the group consisting of the genes listed in table 6, or polypeptide encoded by thereof.

64. A composition for treating or preventing invasion of breast cancer, said composition comprising a pharmaceutically effective amount of an antisense polynucleotide or small interfering RNA against a polynucleotide select from the group consisting of the genes listed in table 5.

65. A composition for treating or preventing invasion of breast cancer, said composition comprising a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by any one gene selected from the group consisting of the genes listed in table 5.

66. A composition for treating or preventing invasion of breast cancer, said composition comprising a pharmaceutically effective amount of the compound selected by the method of any one of claims 52-56 as an active ingredient, and a pharmaceutically acceptable carrier.

67. A method of predicting metastasis of breast cancer, the method comprising the steps of:

(a) detecting an expression level of one or more marker genes in a specimen collected from a subject to be predicted, wherein the one or more marker genes are selected from the group consisting of the genes listed in table 11;

(b) comparing the expression level of the one or more marker genes to that of a metastasis positive case and metastasis negative case; and

(c) when the expression level of the one or more marker genes close to that of a metastasis positive case, is indicative of risk of metastasis of breast cancer, or when the expression level of the one or more marker genes close to that of a metastasis negative case, is indicative of low risk of metastasis of breast cancer.

68. The method of claim 67, wherein step (c) further comprises the steps of calculating a prediction score comprising following steps:

i) calculating the magnitude of the vote (Vi) by the following formula:

$$V_i = | x_i - (\mu_r + \mu_n) /2 |$$

in the fomula; Xi is the expression level in the sample, μ_r is the expression level in the metastasis negative case, and μ_n is the expression level in the metastasis positive case,

ii) calculating PS values by following formula:

$$PS = ((V_r - V_n) / (V_r + V_n)) \times 100$$

in the fomula; V_r and V_n is the total vote of metastasis negative case and metastasis positive case, respectively, and

iii) when the PS values is less than 15.8, determining the subject to be at a risk of having metastasis of breast cancer and when the PS values is more than 15.8, determining the risk of the subject of having metastasis of breast cancer to be low.

69. A breast cancer reference expression profile, comprising a pattern of gene expression of two or more genes selected from the group consisting of the genes listed in table 11.

70. The expression profile of claim 69, wherein the gene expression is derived from a breast cancer cell of a patient with lymph-node metastasis or without lymph-node metastasis.

71. A kit comprising a detection reagent which binds to two or more nucleic acid sequences selected from the group consisting of the genes listed in table 11 or polypeptide encoded thereby.

72. An array comprising two or more nucleic acids which bind to one or more nucleic acid sequences selected from the group consisting of the genes listed in table 11.

73. A method of screening for a compound for treating breast cancer or preventing metastasis of breast cancer, said method comprising the steps of:

(1)contacting a test compound with a polypeptide encoded by a gene selected from the group consisting of VAMP3, MGC11257, GSPT1, DNM2, CFL1, CLNS1A, SENP2, NDUFS3, NOP5/NOP58, PSMD13, SUOX, HRB2, LOC154467, THTPA, ZRF1, LOC51255, DEAF1, NEU1, UGCGL1, BRAF, TUFM, FLJ10726, DNAJB1, AP4S1, and MRPL40:

(2) detecting the binding activity between the polypeptide and the test compound; and

(3) selecting a compound that binds to the polypeptide.

74. A method of screening for a compound for treating breast cancer or preventing metastasis of breast cancer, said method comprising the steps of:

(1) contacting a test compound with a polypeptide encoded by a gene selected from the group consisting of genes listed in table 11;

(2) detecting the biological activity of the polypeptide of step (a); and

(3) selecting a compound that reduces the biological activity of the polypeptide encoded by a gene selected from the group consisting of:VAMP3, MGC11257, GSPT1, DNM2, CFL1, CLNS1A, SENP2, NDUFS3, NOP5/NOP58, PSMD13, SUOX, HRB2, LOC154467, THTPA, ZRF1, LOC51255, DEAF1, NEU1, UGCGL1, BRAF, TUFM, FLJ10726, DNAJB1, AP4S1, and MRPL40 in comparison with the biological activity detected in the absence of the test compound, or elevates the biological activity of the polypeptide encoded by a gene selected from the group consisting of:UBA52, GenBank Acc# AA634090, CEACAM3, C21orf97, KIAA1040, EEF1D, FUS, GenBank Acc# AW965200, and KIAA0475 in comparison with the biological activity detected in the absence of the test compound.

75. A method of screening for a compound for treating breast cancer or preventing metastasis of breast cancer, said method comprising the steps of:

(1) contacting a test compound with a cell expressing one or more marker genes, wherein the marker genes are selected from the group consisting of genes listed in table 11; and

(2) selecting a compound that reduces the expression level of one or more of the marker genes selected from the group consisting of VAMP3, MGC11257, GSPT1, DNM2, CFL1, CLNS1A, SENP2, NDUFS3, NOP5/NOP58, PSMD13, SUOX, HRB2, LOC154467, THTPA, ZRF1, LOC51255, DEAF1, NEU1, UGCGL1, BRAF, TUFM, FLJ10726, DNAJB1, AP4S1, and MRPL40 in comparison with the biological activity detected in the absence of the test compound, or elevates the expression level of one or more of the marker genes selected from the group consisting of UBA52, GenBank Acc# AA634090, CEACAM3, C21orf97, KIAA1040, EEF1D, FUS, GenBank Acc# AW965200, and KIAA0475 in comparison with the biological activity detected in the absence of the test compound.

76. The method of claim 75, wherein said cell expressing one or more marker genes comprises a breast cancer cell.

77. A method of screening for a compound for treating breast cancer or preventing metastasis

of breast cancer, said method comprising the steps of:

(1) constructing a vector comprising the transcriptional regulatory region of a gene selected from the group consisting of genes listed in table 11 upstream of a reporter gene;

(2) transforming a cell with the vector of step (1);

(3) contacting a test compound with the cell of step (2);

(4) detecting the expression of the reporter gene; and

(5) selecting a compound that reduces the expression level of said reporter gene when said marker gene is an up-regulated marker gene selected from the group consisting of VAMP3, MGC11257, GSPT1, DNM2, CFL1, CLNS1A, SENP2, NDUFS3, NOP5/NOP58, PSMD13, SUOX, HRB2, LOC154467, THTPA, ZRF1, LOC51255, DEAF1, NEU1, UGCGL1, BRAF, TUFM, FLJ10726, DNAJB1, AP4S1, and MRPL40. or that enhances the expression level of said reporter gene when said marker gene is a down-regulated marker gene selected from the group consisting of UBA52, GenBank Acc# AA634090, CEACAM3, C21orf97, KIAA1040, EEF1D, FUS, GenBank Acc# AW965200, and KIAA0475, as compared to a control.

78. A method for treating breast cancer or preventing metastasis of breast cancer, said method comprising the step of administering a pharmaceutically effective amount of a compound that is obtained by the method according to any one of claims 73-77.

79. A method for treating breast cancer or preventing metastasis of breast cancer in a subject, said method comprising the step of administering to the subject a pharmaceutically effective amount of an antisense nucleic acids or small interference RNA against one or more genes selected from the group consisting of VAMP3, MGC11257, GSPT1, DNM2, CFL1, CLNS1A, SENP2, NDUFS3, NOP5/NOP58, PSMD13, SUOX, HRB2, LOC154467, THTPA, ZRF1, LOC51255, DEAF1, NEU1, UGCGL1, BRAF, TUFM, FLJ10726, DNAJB1, AP4S1, and MRPL40.

80. A method for treating breast cancer or preventing metastasis of breast cancer in a subject, said method comprising the step of administering to the subject a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by a gene selected from the group consisting of VAMP3, MGC11257, GSPT1, DNM2, CFL1, CLNS1A, SENP2, NDUFS3, NOP5/NOP58, PSMD13, SUOX, HRB2, LOC154467,

THTPA, ZRF1, LOC51255, DEAF1, NEU1, UGCGL1, BRAF, TUFM, FLJ10726, DNAJB1, AP4S1, and MRPL40.

81. A method for treating breast cancer or preventing metastasis of breast cancer in a subject, said method comprising the step of administering to the subject a pharmaceutically effective amount of a polypeptide, polynucleotide encoding thereof or a vector comprising the polynucleotide, wherein the polypeptide is encoded by a gene selected from the group consisting of UBA52, GenBank Acc# AA634090, CEACAM3, C21orf97, KIAA1040, EEF1D, FUS, GenBank Acc# AW965200, and KIAA0475, or the fragment thereof.

82. A method for inducing an anti-tumor immunity, said method comprising the step of contacting with an antigen presenting cell a polypeptide, a polynucleotide encoding the polypeptide or a vector comprising the polynucleotide, wherein the polypeptide is encoded by a gene selected from the group consisting of VAMP3, MGC11257, GSPT1, DNM2, CFL1, CLNS1A, SENP2, NDUFS3, NOP5/NOP58, PSMD13, SUOX, HRB2, LOC154467, THTPA, ZRF1, LOC51255, DEAF1, NEU1, UGCGL1, BRAF, TUFM, FLJ10726, DNAJB1, AP4S1, and MRPL40, or the fragment thereof.

83.The method for inducing an anti-tumor immunity of claim 82, wherein the method further comprises the step of administering the antigen presenting cell to a subject.

84.A composition for treating breast cancer or preventing metastasis of breast cancer in a subject, said composition comprising a pharmaceutically effective amount of a compound that is obtained by the method according to any one of claims 73-77.

85.A composition for treating breast cancer or preventing metastasis of breast cancer in a subject, said composition comprising a pharmaceutically effective amount of an antisense nucleic acids or small interference RNA against one or more genes selected from the group consisting of VAMP3, MGC11257, GSPT1, DNM2, CFL1, CLNS1A, SENP2, NDUFS3, NOP5/NOP58, PSMD13, SUOX, HRB2, LOC154467, THTPA, ZRF1, LOC51255, DEAF1, NEU1, UGCGL1, BRAF, TUFM, FLJ10726, DNAJB1, AP4S1, and MRPL40.

86.A composition for treating breast cancer or preventing metastasis of breast cancer in a subject, said composition comprising a pharmaceutically effective amount of an antibody or fragment thereof that binds to a protein encoded by a gene selected from the group consisting of VAMP3, MGC11257, GSPT1, DNM2, CFL1, CLNS1A, SENP2, NDUFS3,

NOP5/NOP58, PSMD13, SUOX, HRB2, LOC154467, THTPA, ZRF1, LOC51255, DEAF1, NEU1, UGCGL1, BRAF, TUFM, FLJ10726, DNAJB1, AP4S1, and MRPL40.

87. A composition for treating breast cancer or preventing metastasis of breast cancer in a subject, said composition comprising a pharmaceutically effective amount of a polypeptide, a polynucleotide encoding the polypeptide or a vector comprising the polynucleotide, wherein the polypeptide is encoded by a gene selected from the group consisting of VAMP3, MGC11257, GSPT1, DNM2, CFL1, CLNS1A, SENP2, NDUFS3, NOP5/NOP58, PSMD13, SUOX, HRB2, LOC154467, THTPA, ZRF1, LOC51255, DEAF1, NEU1, UGCGL1, BRAF, TUFM, FLJ10726, DNAJB1, AP4S1, and MRPL40, or the fragment thereof.

ABSTRACT

Objective methods for detecting and diagnosing breast cancer (BRC) are described herein. In one embodiment, the diagnostic method involves determining the expression level of BRC-associated gene that discriminates between BRC cells and normal cells. The present invention further provides methods of screening for therapeutic agents useful in the treatment of breast cancer, methods of treating breast cancer and method of vaccinating a subject against breast cancer.

Figure 1

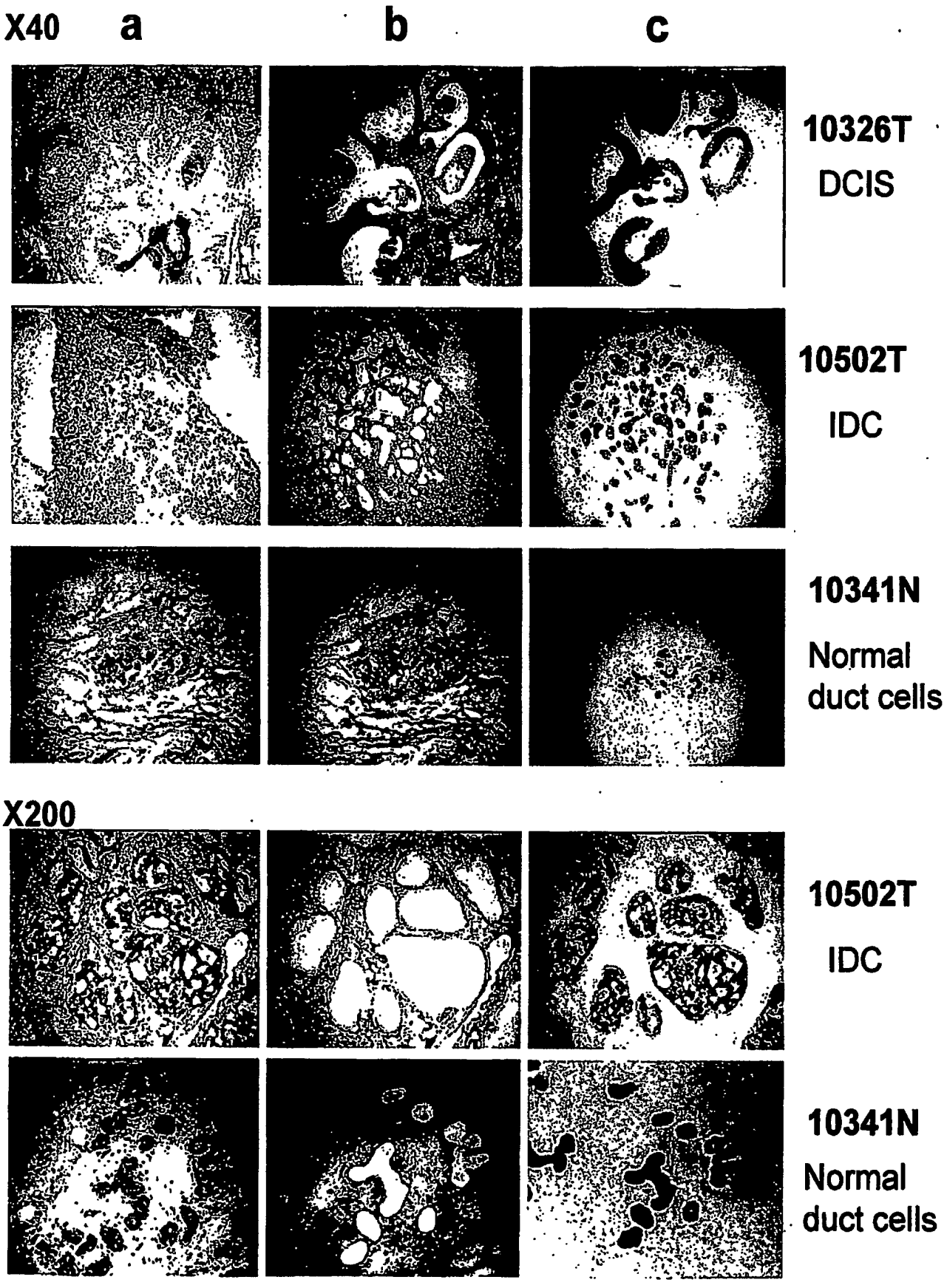

X40
a
b
c
10326T
DCIS
10502T
IDC
10341N
Normal
duct cells
X200
10502T
IDC
10341N
Normal
duct cells

Figure 2A

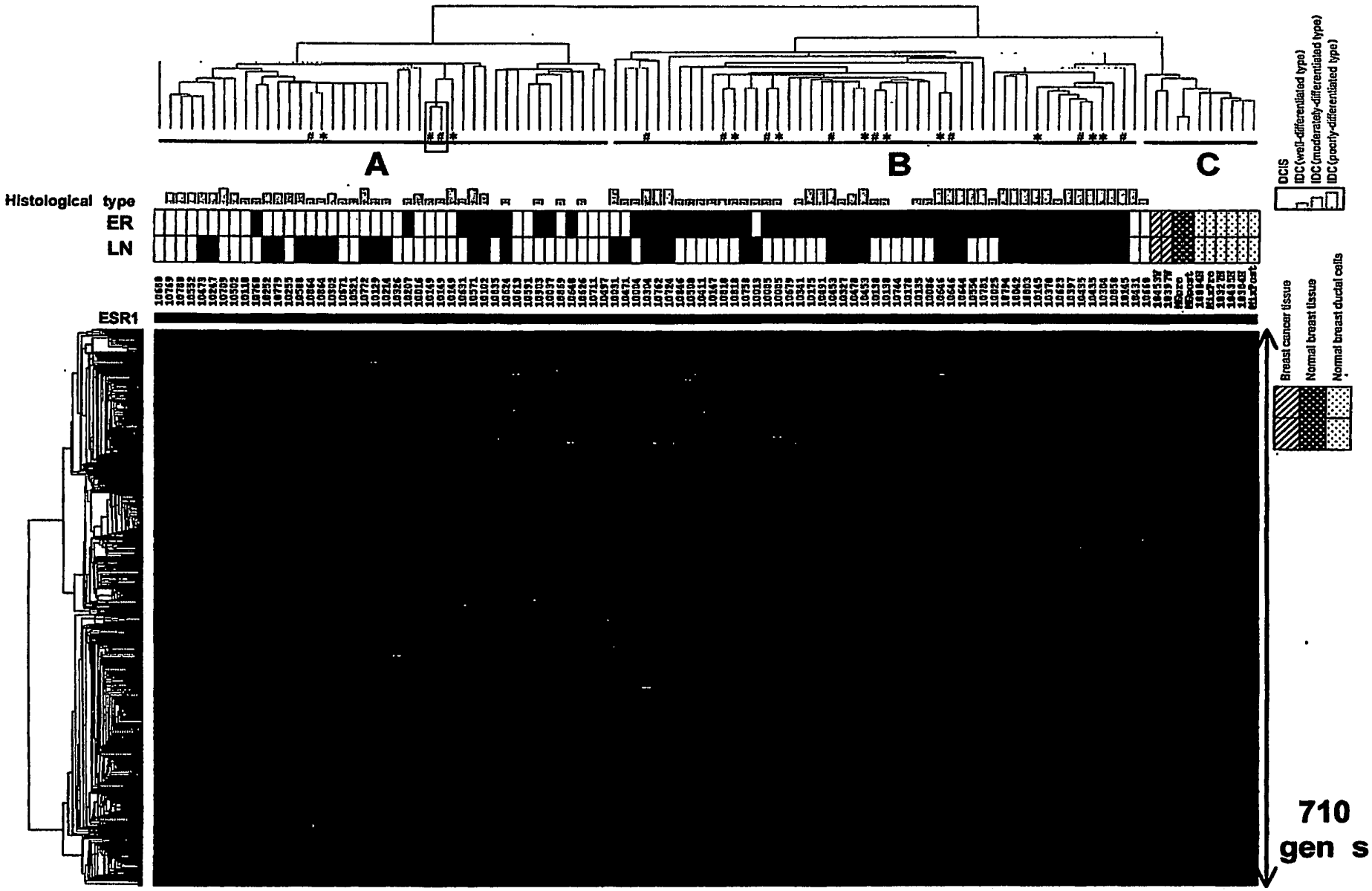

A
B
C
Histological type
ER
LN
ESR1
DCIS
IDC (well-differentiated type)
IDC (moderately-differentiated type)
IDC (poorly-differentiated type)
Breast cancer tissue
Normal breast tissue
Normal breast ductal cells
710
gen s

Figure 2B

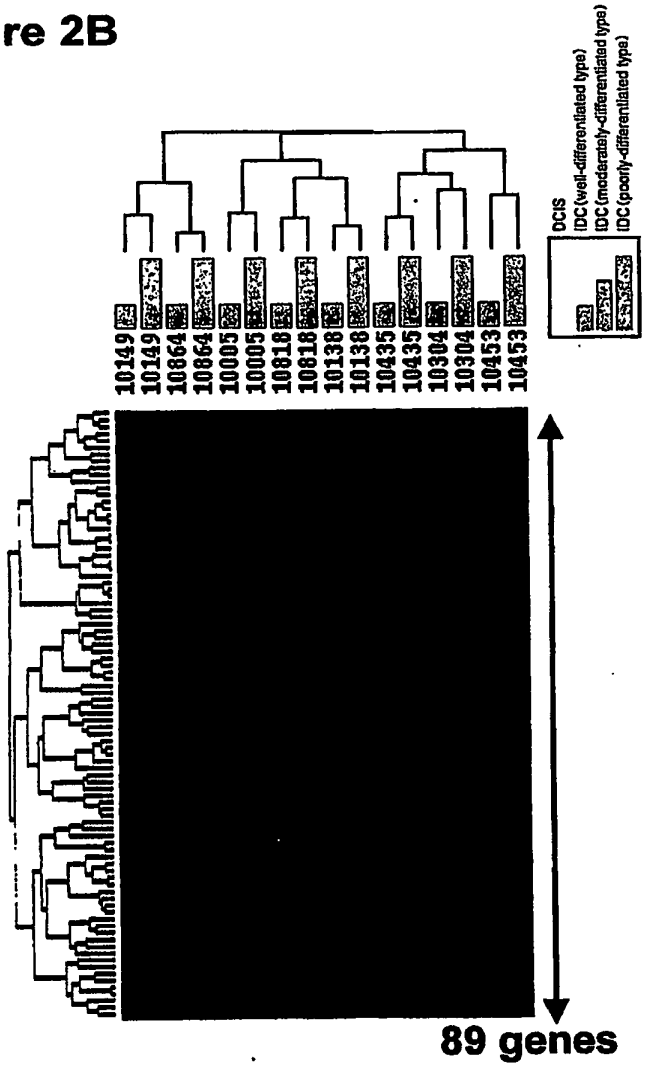


Figure 2C

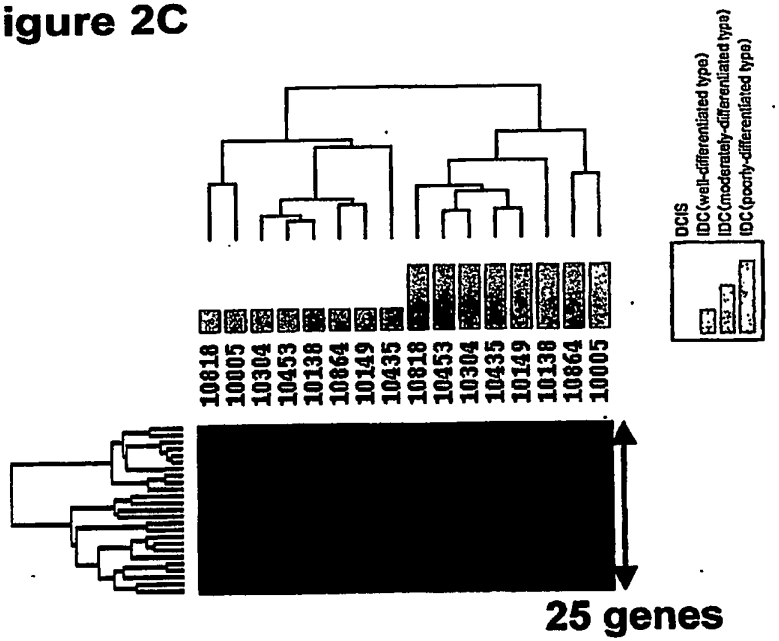


Figure 3

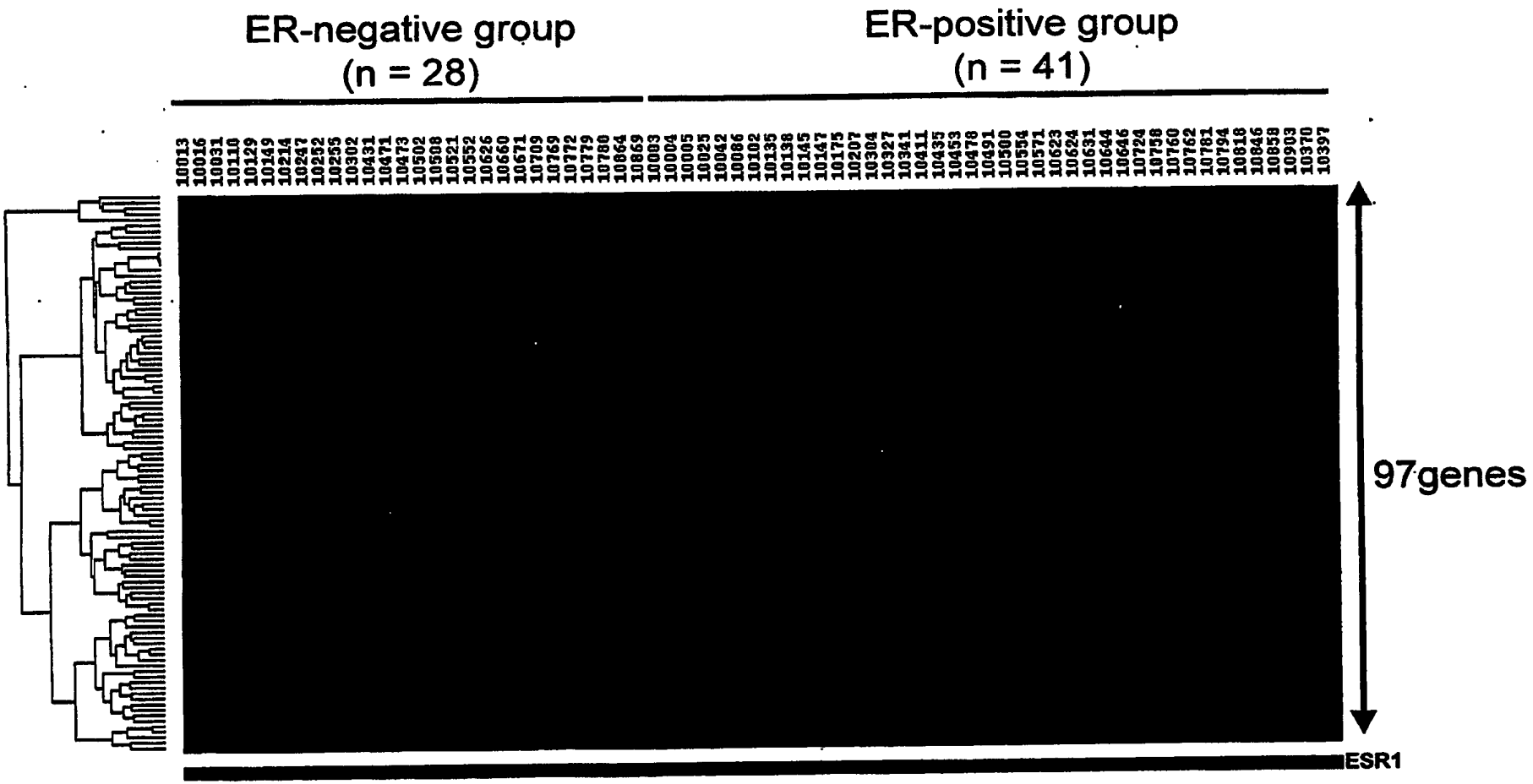

ER-negative group
(n = 28)
ER-positive group
(n = 41)
10013
10016
10031
10110
10129
10149
10214
10247
10252
10255
10302
10431
10471
10473
10502
10508
10521
10552
10626
10660
10671
10709
10769
10772
10779
10780
10864
10869
10003
10004
10005
10025
10042
10086
10102
10135
10138
10145
10147
10175
10207
10304
10327
10341
10411
10435
10453
10478
10491
10500
10554
10571
10623
10624
10631
10644
10646
10724
10758
10760
10762
10781
10794
10818
10846
10858
10903
10370
10397
97genes
ESR1

Figure 4

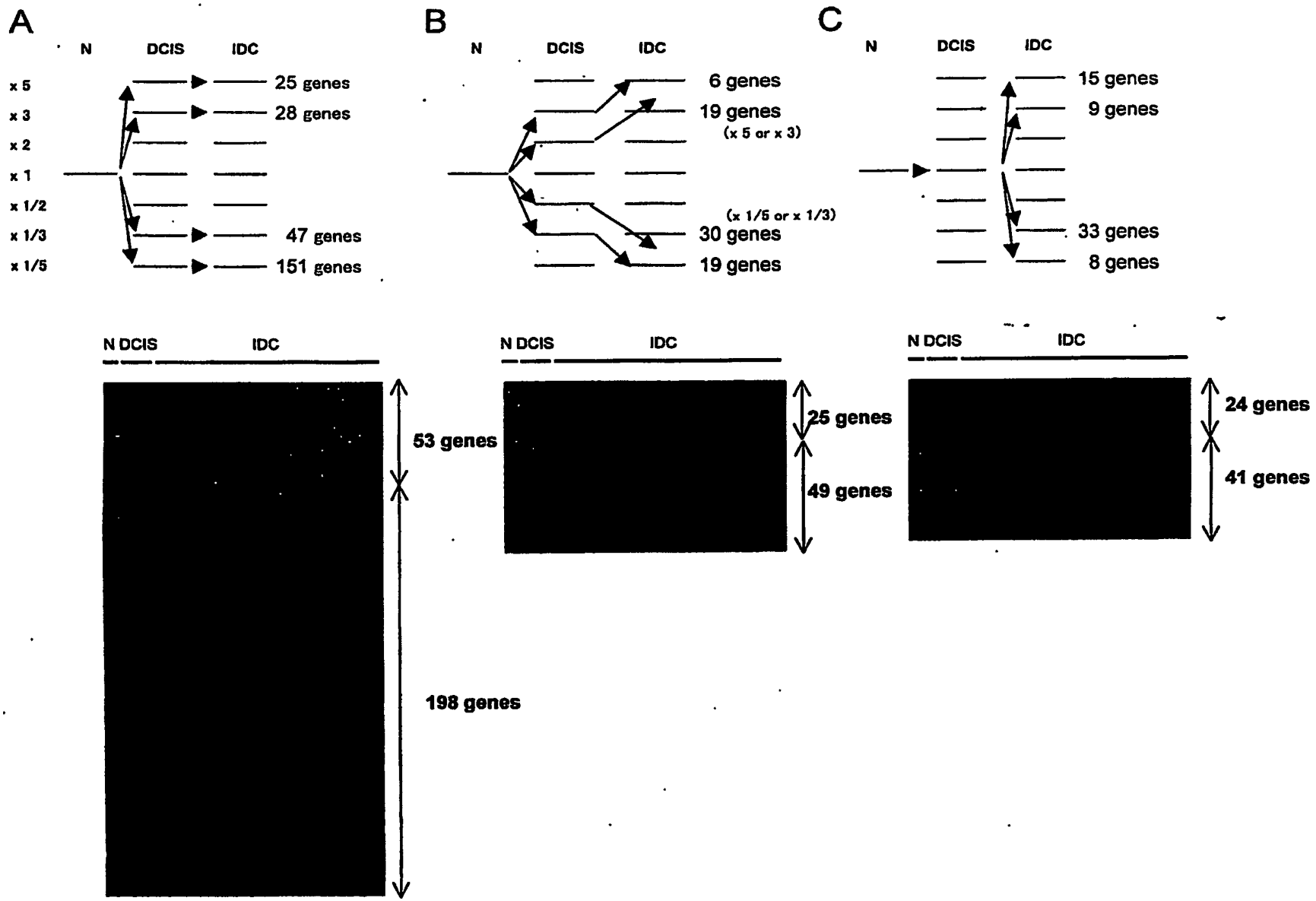

A
N
DCIS
IDC
x 5
x 3
x 2
x 1
x 1/2
x 1/3
x 1/5
25 genes
28 genes
47 genes
151 genes
B
N
DCIS
IDC
6 genes
19 genes
(x 5 or x 3)
(x 1/5 or x 1/3)
30 genes
19 genes
C
N
DCIS
IDC
15 genes
9 genes
33 genes
8 genes
N DCIS
IDC
53 genes
198 genes
N DCIS
IDC
25 genes
49 genes
N DCIS
IDC
24 genes
41 genes

Figure 5

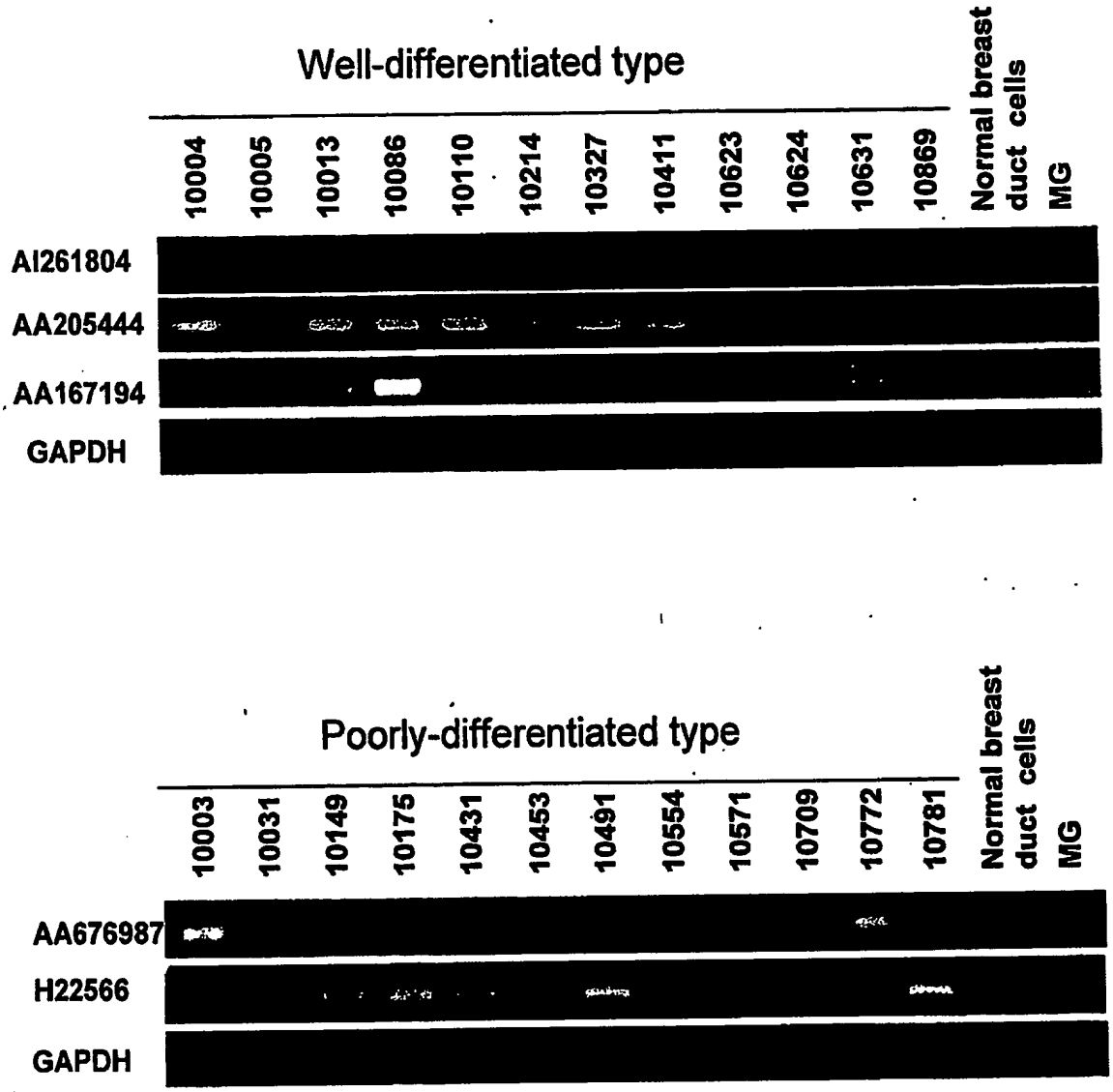

Well-differentiated type
10004
10005
10013
10086
10110
10214
10327
10411
10623
10624
10631
10869
Normal breast duct cells
MG
AI261804
AA205444
AA167194
GAPDH
Poorly-differentiated type
10003
10031
10149
10175
10431
10453
10491
10554
10571
10709
10772
10781
Normal breast duct cells
MG
AA676987
H22566
GAPDH

Figure 6

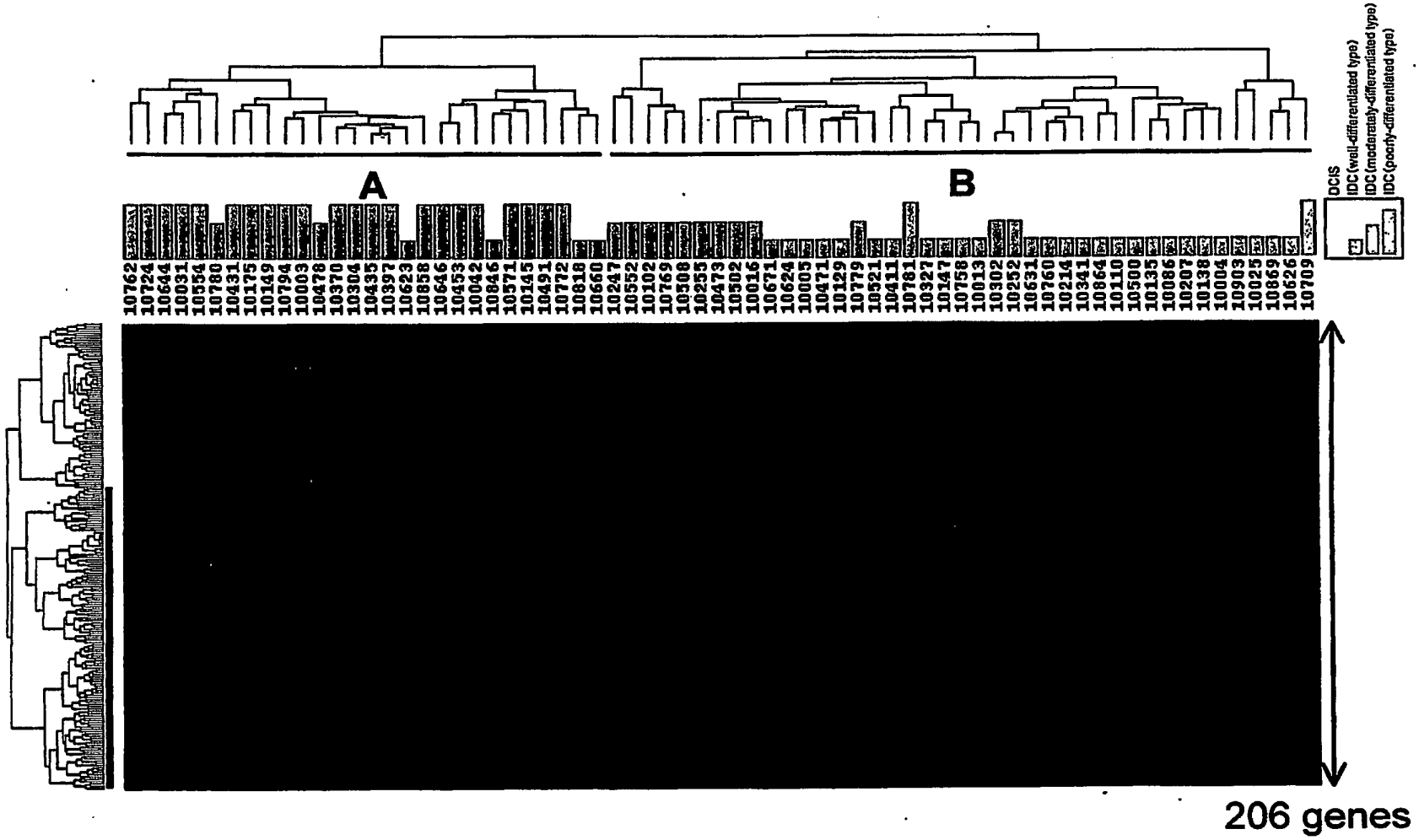
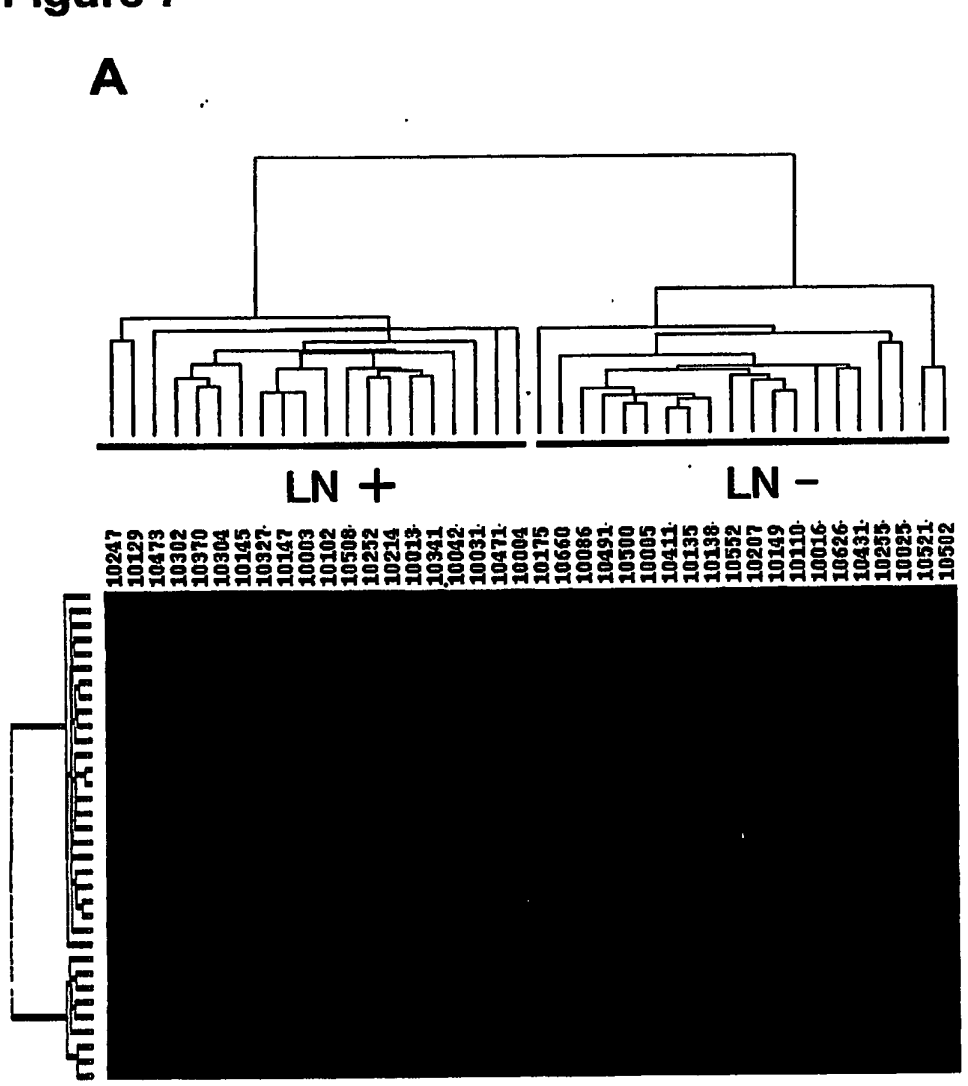

A
B
DCIS
IDC (well-differentiated type)
IDC (moderately-differentiated type)
IDC (poorly-differentiated type)
10762 10724 10644 10031 10554 10780 10431 10175 10149 10794 10003 10478 10370 10304 10435 10397 10623 10858 10646 10453 10042 10846 10571 10145 10491 10772 10818 10660 10247 10552 10102 10769 10508 10255 10473 10502 10016 10671 10624 10005 10471 10129 10779 10521 10411 10781 10327 10147 10758 10013 10302 10252 10631 10760 10214 10341 10864 10110 10500 10135 10086 10207 10138 10004 10903 10025 10869 10626 10709
206 genes

Figure 7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LN +
LN −
10247
10129
10473
10302
10370
10304
10145
10327
10147
10003
10102
10508
10252
10214
10013
10341
10042
10031
10471
10004
10175
10660
10086
10491
10500
10085
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10138
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10149
10110
10016
10626
10431
10255
10025
10521
10502

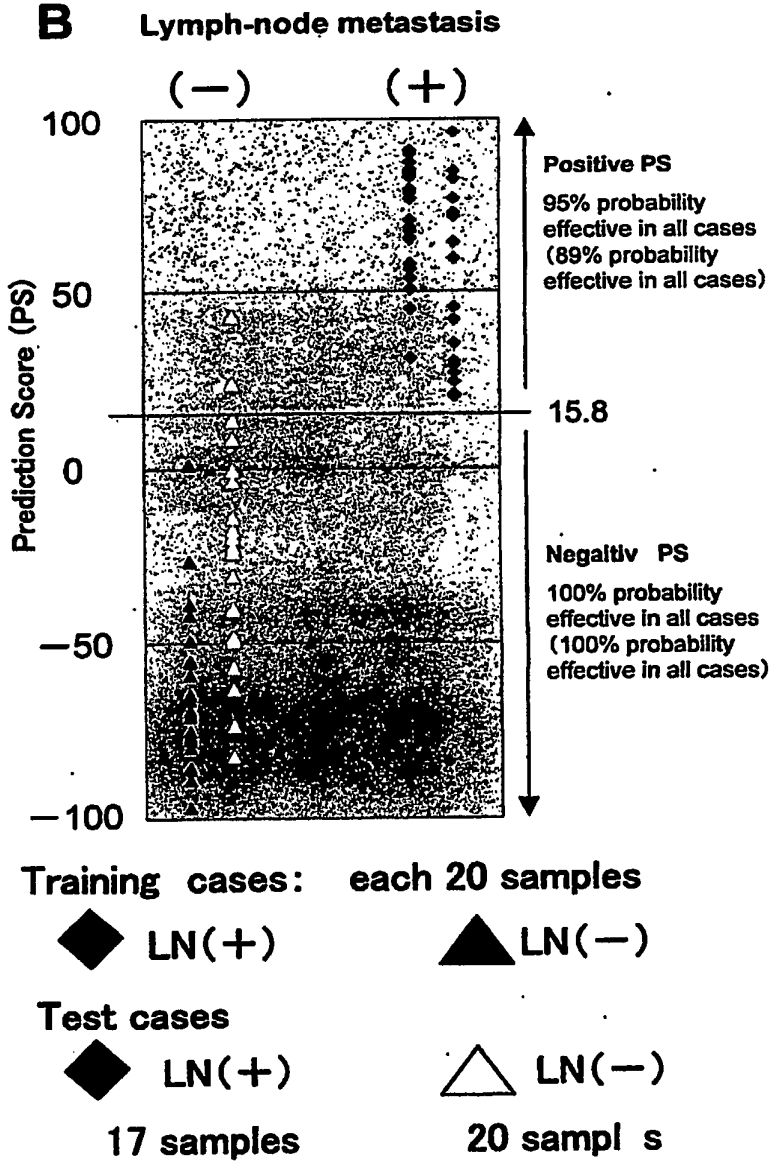
B
Lymph-node metastasis
(−)
(+)
100
50
0
−50
−100
Prediction Score (PS)
Positive PS
95% probability effective in all cases (89% probability effective in all cases)
15.8
Negaltiv PS
100% probability effective in all cases (100% probability effective in all cases)
Training cases: each 20 samples
LN(+)
LN(−)
Test cases
LN(+)
LN(−)
17 samples
20 sampl s

Figure 7C

All Cases

		Lymph-node status		Total
		(+)	(−)	
PS	Positive	37	2	39
	Negative	0	38	38
Total		37	40	77

P<0.001
(χ^2 test for independence)

Test Cases

		Lymph-node status		Total
		(+)	(−)	
PS	Positive	17	2	19
	Negative	0	18	18
Total		17	20	37

P<0.001
(χ^2 test for independence)

SEQUENCE LISTING

<110> NAKAMURA, Yusuke
KATAGIRI, Toyomasa
NAKATSURU, Shuichi

<120> METHOD OF DIAGNOSING BREAST CANCER

<130> ONC-PRV0306-US

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<213> Artificial

<220>
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<210> 5
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